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Biochimica et Biophysica Acta 1508 (2000) 86–111

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Review

Interaction of membrane proteins and lipids with solubilizing detergents

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Abstract

Detergents are indispensable in the isolation of integral membrane proteins from biological membranes to study their intrinsic structural and functional properties. Solubilization involves a number of intermediary states that can be studied by a variety of physicochemical and kinetic methods; it usually starts by destabilization of the lipid component of the membranes, a process that is accompanied by a transition of detergent binding by the membrane from a noncooperative to a cooperative interaction already below the critical micellar concentration (CMC). This leads to the formation of membrane fragments of proteins and lipids with detergent-shielded edges. In the final stage of solubilization membrane proteins are present as protomers, with the membrane inserted sectors covered by detergent. We consider in detail the nature of this interaction and conclude that in general binding as a monolayer ring, rather than as a micelle, is the most probable mechanism. This mode of interaction is supported by neutron diffraction investigations on the disposition of detergent in 3-D crystals of membrane proteins. Finally, we briefly discuss the use of techniques such as analytical ultracentrifugation, size exclusion chromatography, and mass spectrometry relevant for the structural investigation of detergent solubilized membrane proteins.

Keywords: Detergent; Surfactant; Solubilization; Monolayer; Membrane protein; Structure

1. Introduction

Detergents are indispensable as solubilizing agents in the isolation and purification of membrane proteins [1–7]. Detergents permit us to study the detailed properties of membrane proteins in protomeric and self-associated states as well as in their interactions with other proteins. Detergent solubilization is also required as a first step for reconstitution and in attempts at membrane protein crystallization. For the correct use of detergents it is necessary to have an idea of how, and in which amounts, they interact with integral membrane proteins and membrane lipid. These are the main topics that we attempt to cover in this review, at the same time bearing in mind that solubilization normally aims at the preservation of both structural and functional properties, by no means a simple issue. In Section 2 of this review we focus on the solubilization of membranes

Abbreviations: C_{10}DAO, decyldimethyl-*N*-amineoxide; $C_{12}E_8$, octaethylene glycol monododecyl ether; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CMC, critical micelle concentration; DDAO, dodecyldimethyl-*N*-amineoxide; CSA, cross-sectional area; DM, dodecyl-β-D-maltoside; DDMAB, *N*-dodecyl-*N,N*-(dimethylammonio) butyrate; DOPC, dioleoylphosphatidylcholine; DPC, dodecylphosphocholine; HECAMEG, 6-O-(*N*-heptylcarbamoyl)-methyl-β-D-glucopyranoside; HPLC, high performance liquid chromatography; LAPAO, lauramido-*N,N*-dimethyl-3-*n*-propylamineoxide; OG, octyl-β-D-glucoside; SR, sarcoplasmic reticulum

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0005-2736/00/$ – see front matter
PII: S0304-4157(00)00010-1

and membrane proteins, after a brief overview of detergents often used for this purpose. In Section 3 we examine in detail models concerning the mode of detergent interaction with membrane proteins. Finally, in Section 4 we briefly consider some methods which are useful in the characterization of detergent solubilized membrane proteins with respect to their molecular mass, size, and other structural parameters.

2. Solubilization of biological (protein containing) membranes

2.1. Detergents commonly used for solubilization of membrane proteins

Tables 1 and 2 summarize physicochemical characteristics (critical micellar concentrations (CMC), aggregation numbers and partial specific volumes) of polyoxyethyleneglycol and other detergents often used in the study of membrane proteins. This information is important for the use of the detergents in solubilizing and purifying membrane proteins for structural studies. With respect to the size of micelles it may be noted that well defined micelles, shaped as prolate or, more likely, oblate ellipsoids of revolution [15,22,60] or spheres [61] can be expected to have aggregation numbers around 50–100 (see also Parades and Tribout in this issue and the discussion in [192]). As can be seen, most of the detergents shown in Tables 1 and 2 have aggregation numbers which fall within this range, or nearly so. It should be mentioned that aggregation numbers and, to a lesser extent, CMCs, are difficult to measure with great accuracy so that the values found in the literature are sometimes quite variable. In addition these parameters can be affected by experimental conditions, so that in critical cases it is recommended that investigators perform these measurements themselves rather than rely on tables (see e.g., [30,196,197]). Here, we only briefly comment on one particular detergent, viz. Triton X-100: by sedimentation equilibration centrifugation we found an aggregation number for this detergent of 75, while other investigators with different techniques report aggregation numbers twice as high [22]. Lamellar sheets, similar to those formed by soaps [12], may be formed by long chain detergents, but so far there is little evidence (with the exception of Tween-80 [21,62]) of the usefulness of such detergents for maintenance of activity in their interaction with membrane proteins.

Polyoxyethylene- and other non-ionic detergents are generally 'mild', i.e., we can usually assume that they solubilize membrane proteins without affecting important structural features. Nevertheless, solubilization by many detergents frequently leads to inactivation; this is particularly the case for detergents with a short (C_7–C_{10}) hydrocarbon chain, e.g., octylglucoside and C_8E_4 or C_8E_5 which are often more inactivating than corresponding detergents with an intermediary (C_{12}–C_{14}) hydrocarbon chain length [43]. The short-chain detergents have found use in the crystallization of e.g., reaction center [63] and porin [59]. An additional interest of C_8E_5 is that its $\bar{v}_D$ is close to unity (see Table 1) so that its mass contribution can be neglected in sedimentation equilibrium experiments [11,44]. Other alkylglucosides than octylglucoside, in particular β-dodecylmaltoside (DM), have been increasingly used in solubilization of membrane proteins with retention of functional properties [29,43,44].

Zwitterionic detergents (DDAO, LAPAO, zwittergents (sulfobetaines), etc., cf. Table 2), comprise a heterogeneous group of compounds which in general is more inactivating than non-ionic detergents. However, in selected cases they can act as substitutes of polyoxyethylene detergents in connection with structural studies of membrane proteins; as examples it can be mentioned that DDAO has been used for crystallization of reaction center [45] and for structural studies on mammalian rhodopsin [32]. Note that DDAO is uncharged at pH 5 and above while it is positively charged at low pH, a change which of course modifies its properties (e.g., [198,199] and refs. therein). DDMAB is an efficient solubilizer of membrane proteins (e.g., [46]) and has been used in a small-angle X-ray scattering study of glycophorin because its average electron density matches that of the buffer [47]. This means that the contribution of bound detergent to the small-angle scattering vanishes, while in previous experiments with this technique (rhodopsin in DDAO [32]; Ca^{2+}-ATPase in deoxycholate [48]; reaction center in DDAO [49]) contrast variation with sucrose had to be performed

Table 1
Properties[a] of polyoxyethylene glycol detergents (updated from [7])

	Monomer mass (M_r)	CMC (M)	Aggregation number	$\bar{v}_D$ (cm^3/g)	Ref.[b]
Homogeneous compounds[c]					
C_8E_4	306	$7–8.5\times10^{-3}$	82	–	[8–10]
C_8E_5	350	$4.3–9.2\times10^{-3}$	–	0.993[e]	[8–11]
C_8E_6	394	1×10^{-2}	32	0.963	[12–14]
$C_{10}E_6$	422	9×10^{-4}	73	–	[12]
$C_{12}E_6$	450	8.2×10^{-5}	105[d]	0.989	[2,12,14]
$C_{12}E_8$	538	9×10^{-5}	90–120	0.973	[2,15–17]
$C_{16}E_6$	506	1.3×10^{-6}	2400	–	[12]
$C_{16}E_9$	638	2.1×10^{-6}	280	–	[12]
$C_{16}E_{12}$	770	2.3×10^{-6}	150	–	[12]
$C_{16}E_{21}$	1166	3.9×10^{-6}	70	–	[12]
p-tert-$C_8\varnothing E_9$	602	3.0×10^{-4}	–	–	[12]
$C_9\varnothing E_{10}$	676	7.5×10^{-5}	–	–	[18]
Heterogeneous compounds[c]					
$C_{12\&14}E_{\langle9.5\rangle}$ (Lubrol PX)	620	1×10^{-4}	100	0.958	[2,12,19,20]
$C_{12}E_{\langle12\rangle}$	710	9×10^{-5}	80	–	[2,12]
$C_{12}E_{\langle23\rangle}$ (Brij 35)	1200	9×10^{-5}	40	–	[12]
$C_{16\&18}E_{\langle17\rangle}$ (Lubrol WX)	1000	4×10^{-6}	90	0.929	[2,15,21]
p-tert$C8\varnothing E_{\langle9.5\rangle}$ (Triton X-100)	625	2.5×10^{-4}	75–165	0.908	[16,17,22,23]
p-tert-$C8\varnothing E_{\langle7–8\rangle}$ (Triton X-114)	540	2×10^{-4}	–	0.869	[22,23]
$C9\varnothing E_{\langle10\rangle}$ (Triton N-101)	670	1×10^{-4}	100	0.922	[13,24]
C_{12}sorbitan $E_{\langle20\rangle}$ (Tween-20)	1240	6×10^{-5}	–	0.869	[12,23]
$C_{18:1}$sorbitan $E_{\langle20\rangle}$ (Tween-20)	1320	$0.7–1.2\times10^{-5}$	60	0.896	[12,21,23,25]

[a]Data obtained at 20–25°C by physicochemical methods (surface tension, light scattering, densitometry, analytical ultracentrifugation, fluorescence). Salts should not affect much CMC or aggregation number of non-ionic detergents (however, see [196]).
[b]The references indicated are either for the original data or for data surveys.
[c]Nomenclature: C_xE_y: x refers to the number of C atoms in the alkyl chain and y to the (average) number of polyoxyethylene glycol units; $\varnothing$ denotes a phenyl group. Commonly used trade names are indicated in parentheses.
[d]Measured at 4°C, because of secondary aggregation at 25°C.
[e]Measured for a mixture of C_8E_4 and C_8E_5 [11].

to take into account the effect of the bound detergent. This was especially cumbersome in the case of reaction center, because sucrose decreased detergent binding and promoted protein aggregation [49]. Finally, it is interesting to mention that *non-detergent* sulfobetaines have been successfully used as mild solubilization agents in conjunction with detergents for protein purification [191].

Ionic detergents such as sodium dodecyl sulfate (SDS) are efficient solubilizers, but almost always denaturing. However, SDS is frequently being used as a membrane mimetic environment in NMR studies on transmembrane peptides and the involvement of electrostatic interactions in the mechanism of peptide folding induced by SDS binding has been described [200]. In some cases reactivation of SDS solubilized proteins is possible [64,65]. Based on the pioneering work of Lauterwein et al. [36] dodecylphosphocholine (DPC) is finding increasing use in NMR experiments. This detergent is an efficient solubilizer of hydrophobic or amphipathic α-helices (see, e.g., [50,51] and references therein). In addition the aggregation number of DPC is low so that it is possible to obtain high-resolution NMR spectra. It has been shown that the dynamic behavior of the phosphocholine groups of DPC at low temperatures (12°C) corresponds to that in a phosphatidylcholine membrane–water interface above its melting temperature [51]. The properties of short-chain phospholipids (e.g., di-$C_{6:0}$PC or di-$C_{7:0}$PC) which are interesting alternatives to DPC or to other detergents are dealt with in another chapter of this issue (see Hauser, this issue; [194,195]).

Steroid-based compounds like bile salts and

Table 2
Properties[a] of various types of polar or non-ionic detergents, and of bile salts (updated from [7])

	Monomer mass (M_r)	CMC (M)	Aggregation number	$\bar{v}_D$ (cm^3/g)	Ref.[b]
Octyl-β-D-glucoside (OG)	292	$1.9–2.5\times10^{-2}$	≈90	0.859	[8,20,26,27,192]
Decyl-β-D-maltoside	483	2.2×10^{-3}	–	–	[28]
dodecyl-β-D-maltoside (DM)	511	1.8×10^{-4}	110–140	0.81–0.837	[17,20,26,29,192]
Cyclohexyl-hexyl-β-D-maltoside (CYMAL-6)	509	5.6×10^{-4}	63[f]	–	[57]
2-*O*-Lauroylsucrose	524	6.5×10^{-4}	–	–	[30]
Dodecyldimethyl-*N*-amineoxide (DDAO)	229	2.2×10^{-3}	69–73	1.128–1134	[17,31,32,199]
Lauroamido-*N*,*N*-dimethyl-3-*n*-propylamineoxide (LAPAO)	302	3.3×10^{-3}	–	1.067	[33]
Dodecyl-*N*-sulfobetaine (zwittergent 3-12)[c]	336	$1.4–4\times10^{-3}$	55–87	–	[6,20]
Tetradecyl-*N*-sulfobetaine (zwittergent 3-14)	364	$1–60\times10^{-4}$	83–130	–	[6,19]
N-dodecyl-*N*,*N*-(dimethylammmonio) butyrate (DDMAB)	300	4.3×10^{-3}	47	1.07	[34,35]
1-Myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholoine ($C_{14:0}$lysoPC)	468	9×10^{-5}	–	0.97	[6,13]
1-Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine ($C_{16:0}$lysoPC)	496	1×10^{-5}	–	0.976	[6,13]
N-dodecylphosphocholine (DPC)	352	1.1×10^{-3}	50–60	0.937	[36,215]
1,2 Diheptanoyl-*sn*-glycero-3-phosphocholine (di-$C_{7:0}$PC)	482	$1–1.4\times10^{-3}$	42–200[g]	0.888–0.925	[5,13,58,59]
3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)[c]	615	$3–10\times10^{-3}$	4–14	0.81	[19,20,37]
Deoxycholic acid[d,e]	393	3×10^{-3}	22	0.778	[3,13]
Cholic acid[d,e]	409	1×10^{-2}	4	0.771	[13,38]
Taurodeoxycholic acid[d]	500	1.3×10^{-3}	20	0.75	[13,38]
Glycocholic acid[d]	466	–	6	0.77	[13,38]
Sodium dodecylsulfate[c]	288	$1.2–7.1\times10^{-3}$	62–101	0.863	[5,19,20,39]
6-*O*-(*N*-heptylcarbamoyl)-methyl-β-D-glucopyranoside (HECAMEG)	335	1.95×10^{-2}	92	–	[40,192]

[a]Data obtained at 20–25°C, pH ≈ 7.
[b]The references indicated are either for the original data or for data surveys.
[c]The lower values of CMC are obtained at 0.1–0.2 M Na^+, the higher values at 0–0.05 M Na^+ (data surveyed by [19,20]). For SDS, the $\bar{v}_D$ is lower below the CMC [39].
[d]Data refer to $\mu = 0.15$, pH 8.0 (micellar properties are strongly affected, in particular by changes in ionic strength).
[e]Data for CMC refer to the authors' own measurements by the dye uptake method [41].
[f]An Anatrace Inc. measurement.
[g]Not well-defined micelles [58].

CHAPS form a separate class of relatively mild detergents, characterized by low and ionic strength dependent aggregation numbers. The use of these compounds quite often leads to less inactivation than hydrocarbon detergents with the same hydrophilic head groups (e.g., by CHAPS compared to Zwittergents [43]). Like octylglucoside, steroid detergents are often used for reconstitution because of a high CMC. On the other hand, they have been unsuccessful in, e.g., the crystallization of porin [59].

Besides the detergents shown in Tables 1 and 2 some new detergents have been synthesized for specific purposes, for example brominated dodecylmaltoside and lauroylsucrose, which have fluorescence quenching properties [30,201]. In particular, 7,8-dibromododecylmaltoside (Br-DM) has proved to be a useful tool for the study of protein detergent interactions during the different steps of solubilization of sarcoplasmic reticulum membranes [30] and as an aid to follow the changes of structure (movement of helix no. X) associated with ligand binding to lactose permease [52]. More recently, we have used this com-

pound to analyze the propensity of Ca^{2+}-ATPase peptides to form transmembrane segments from their interaction with detergent micelles and to investigate the position of specific tryptophan residues within detergent micelles and hence, by analogy, their location within the native membrane [53]. Perfluorinated alkanes are poorly miscible with alkanes so the use of fluorinated detergents were tested as they ought to be less efficient in removing the protein lipids and other hydrophobic factors or to separate oligomers [54]. Some of them are potentially interesting membrane solubilizing agents [54–56,159], but the solubilization efficiency is often low [54,55,159]. A method analogous to SDS–polyacrylamide gel electrophoresis (PAGE) has been described using perfluoro-octanoic acid for the evaluation of the oligomeric structure of membrane proteins [56].

In summary, the choice of the appropriate detergent remains a difficult task but increasing experience can help to focus on a given set of detergent for a given purpose (e.g., for solubilization versus crystallization or reconstitution). A few detergents like $C_{12}E_8$ or dodecylmaltoside are apparently of rather general use for membrane proteins, being appropriate choices in many solubilization and crystallization studies, as well as in reconstitution experiments. Indeed reconstitution experiments no longer require high CMC detergents since it has been shown that, by the use of polystyrene beads, it is possible to remove efficiently and in a short time detergents with low CMC (see, e.g., [97] and Rigaud et al. in this issue).

2.2. Solubilization of the lipid component

The solubilization of pure phospholipid membranes by detergents and the interactions within such detergent–phospholipid systems have been the subject of several investigations (see reviews by Lichtenberg, Walter, Ollivon et al., Almgren and Edwards, Heerklotz and Seelig, in this volume). The data obtained on such systems with homogenous phospholipids are usually analyzed in terms of a sharp phase transition of lipid from a membranous to a micellar state according to the three stage hypothesis: In Stage I non-micellar detergent partitions into the phospholipid bilayer, whereas in Stage III phospholipid is fully solubilized by uptake into detergent micelles. In the intermediary Stage II phospholipid membranes saturated with incorporated detergent are assumed to coexist at thermodynamic equilibrium with mixed phospholipid–detergent micelles saturated with phospholipid. To extrapolate from this simple situation to the solubilization of complex biological membranes we have compared the interactions of efficiently solubilizing detergents with (i) such homogeneous phospholipid systems (dioleoylphosphatidylcholine, DOPC) (ii) Ca^{2+}-ATPase membranes (prepared from SR vesicles) and (iii) liposomes prepared from SR lipid [66,67]. In these experiments detergent was added in different amounts to the various membrane preparations and the concentration of free detergent in the resulting mixture evaluated by equilibrium dialysis. We found that at low concentrations the incorporation of polyoxyethyleneglycol detergents ($C_{12}E_8$, Triton X-100), dodecylmaltoside, and DDAO into the membranes followed hyperbolic isotherms which, however, before the approach to saturation were interrupted by the onset of a strongly cooperative binding process. This breakpoint occurred below the CMC for unbound detergent, as demonstrated in the case of $C_{12}E_8$ in Fig. 1. For a binary mixture of $C_{12}E_8$ and pure DOPC, solubilization (corresponding to Stage II) takes place in a very narrow range of concentrations of unbound detergent, whereas for both Ca^{2+}-ATPase membranes and SR-lipid liposomes the free detergent concentration during solubilization rises slightly and tends towards the CMC for pure detergent. The constant concentration of $C_{12}E_8$ during solubilization of pure DOPC is in agreement with the three-stage hypothesis by indicating the presence of thermodynamic equilibrium between only two phases, the solubilized and non-solubilized phases, whereas the increase in free $C_{12}E_8$ associated with progressive solubilization of both Ca^{2+}-ATPase and SR-lipid membranes probably is caused by the heterogeneous nature of their lipid composition (presumably mainly with respect to differences in the acyl chain composition). Another notable feature of Fig. 1 is that there is no difference between the binding isotherms of the Ca^{2+}-ATPase and SR-lipid preparations, if binding is expressed in terms of moles of detergent incorporated per mole of lipid. This is an indication that the bulk of added detergent interacts with membrane lipid, and not with protein,

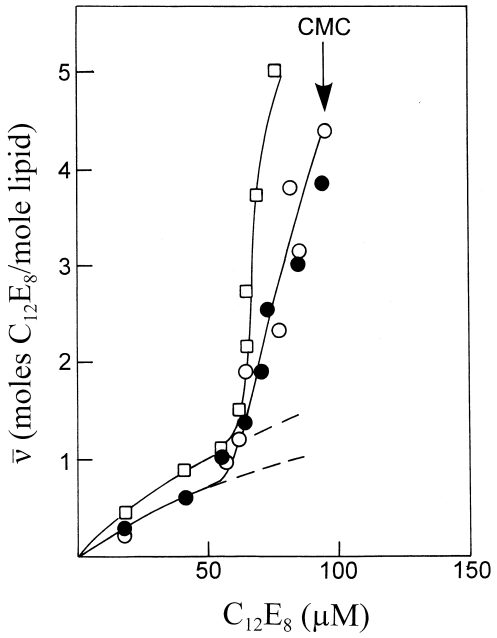

Fig. 1. Binding of $C_{12}E_8$ by Ca^{2+}-ATPase, SR-liposomes, and pure dioleoylphosphatidylcholine membranes. The experiments were performed by equilibrium dialysis over 48–72 h in *cis* experiments where detergent initially was present together with membranes in the same compartment of the cell. The symbols refer to the following: Ca^{2+}-ATPase membranes (●), 0.5 mg protein/ml and 0.25 mg lipid/ml; SR liposomes (○), 0.25 mg lipid/ml; and DOPC unilamellar liposomes (□), 0.25 mg lipid/ml. Data taken from Kragh-Hansen et al. [67] with permission.

during incorporation into the Ca^{2+}-ATPase membranes before solubilization.

A somewhat different description of the solubilization process than discussed above has been given by Rigaud and collaborators who studied the interaction of $C_{12}E_8$, Triton X-100, octylglucoside, and cholate with liposomal preparations, consisting of 90% egg yolk lecithin and 10% phosphatidic acid [202,203]. In most cases the extent of interaction was estimated indirectly on the basis of light scattering data obtained at various liposomal concentrations. In the study by Levy et al. [203] unbound $C_{12}E_8$ was estimated by subjecting the liposomal samples to a strong centrifugal field to separate a sediment of $C_{12}E_8$ containing liposomes from unbound $C_{12}E_8$ and $C_{12}E_8$–lipid micelles that were assumed to remain in the supernatant. The data were described by a partition coefficient, assuming that in Stage I detergent uptake by the lipid phase is proportional to the concentration of free $C_{12}E_8$ up to the point of solubilization (Stage II). The amount of detergent bound to the sediment in Stage II was considered to represent a situation where the membranes had become saturated by detergent (notice that according to our analysis, as shown by the broken lines in Fig. 1, solubilization only occurs because of a transition from noncooperative to cooperative interaction, and not because the membranes in a strict sense have become saturated by detergent, see also the recent paper by Heerklotz and Seelig [204] on the differences in uptake accompanied by solubilization of liposomes with strong and mild detergents). In our experiments proportionality between bound and free detergent can only be considered to be an adequate description of the data at low detergent concentrations, corresponding to the start of Stage I, resulting in a gradual, rather than abrupt, decline in liposomal detergent binding at the transition between Stage I to Stage II [66,67]. This is not surprising, since the composition and properties of the lipid phase change appreciably [205] during the uptake in phase I (which encompasses incorporations of levels from close to zero to about 0.7 moles of $C_{12}E_8$ per mole of lipid). Similar considerations apply to other detergents, where physicochemical properties such as lipid fluidity [206,207], static order of the lipid hydrocarbon chains [208,209] and liposomal leakiness [202,210] change appreciably even by incorporation of small amounts of detergent into the membrane.

The characteristic features of the solubilization process as discussed above apply to detergents that efficiently solubilize biological membranes without causing fundamental changes in the structure of the solubilized membrane protein. With the more aggressive SDS we observed interaction with the protein in Ca^{2+}-ATPase membranes not only at high, but also at low detergent concentrations, before denaturation of the protein [67]. With regard to the state of the lipid in Ca^{2+}-ATPase membranes it is generally assumed, on the basis of ESR and NMR spectroscopic evidence [68], that lipid in contact with Ca^{2+}-ATPase (boundary lipid) is in a relatively immobilized state, compared to lipid in the Ca^{2+}-ATPase membranes further removed from the protein or to lipid in pure bilayers. However, competition experiments between different lipid species [69] and analysis of the lipids remaining bound to Ca^{2+}-ATPase after detergent solubilization [25,70] have failed to reveal any

difference in affinity for binding of lipid in the boundary region of Ca^{2+}-ATPase or SR membranes (note, however, opposite results in [160,161]). Furthermore, it has been demonstrated by the use of brominated detergents that these compounds even at low non-solubilizing concentrations efficiently interact with Ca^{2+}-ATPase, as evidenced by their ability to quench by contact the tryptophan residues present at the hydrophobic surface of Ca^{2+}-ATPase [30]. Lipids surrounding Ca^{2+}-ATPase thus do not appear to act as a barrier for contact between detergent and the membrane inserted part of the membrane protein. In fact, detergent and the various lipid species present in Ca^{2+}-ATPase membranes appear to be fairly uniformly distributed over the whole lipid phase before the onset of solubilization.

Although cooperative binding is a hallmark of solubilization by non-ionic detergents it should be noted that these two events are not exactly coincident in that the onset of cooperative binding in most cases slightly precedes solubilization of protein and lipid. This was observed first with dodecylmaltoside [66] and was later found also to be a feature of the interaction of Triton X-100 and DDAO with Ca^{2+}-ATPase membranes [67]. In agreement with this conclusion, Lopez et al., have recently published cryoEM pictures which were interpreted to indicate the presence of micellar structures of Triton X-100 within liposomal membranes immediately before the onset of solubilization [71]. For $C_{12}E_8$ we observed that lipid was solubilized before the protein and that this process was concomitant with the onset of cooperative binding [67].

For pure lipid membranes, without protein, we find that fusion of small vesicles to larger vesicles is a characteristic response to the onset of cooperative binding [67]. Such fusion has also been reported by other authors upon addition of $C_{12}E_8$ [72], Triton X-100 [73] and octylglucoside [74,75]. For octylglucoside [74] and cholate [76] a rich variety of intermediary structures, comprising open vesicles, membrane sheets, and elongated cylindrical micelles have been observed by cryotransmission electron microscopy (CTEM). In addition, by solubilization of liposomes with dodecylmaltoside near the Stage II to Stage III transition the solution assumes viscous gel-like properties which according to CTEM appear to arise from the formation of a filamentous network of long cylindrical micelles [83,211]. On the other hand, solubilization by Triton X-100 seems to proceed without the formation of many intermediary forms, and this property has been suggested to be the basis for unidirectional and more efficient liposomal reconstitution of the solubilized lactose transporter with Triton X-100 by Biobeads treatment than was possible in similar experiments with the use of dodecylmaltoside as a solubilizing agent [83]. A limitation in the use of dodecylmaltoside for reconstitution is also suggested by the study of Lambert et al. [211] who observed that slow removal of dodecylmaltoside with Biobeads from fully solubilized lipid samples resulted in the formation of many multilamellar vesicles. However upon rapid removal almost unilamellar liposomes were produced in this detergent [211].

For Ca^{2+}-ATPase membranes we observed that, in contrast to lipid membranes, the intermediary stages of the solubilization process with dodecylmaltoside was different in that in that it only appeared to result in the formation of bilayer containing membrane fragments in Stage II that, however, were prone to undergo secondary aggregation at room temperature [66]. We may envision that these fragments represent structures stabilized at the edges by a semitoroidal ring of assembled detergent, similar to the models used to explain solubilization of protein containing membranes by bile salt detergents [38,77,78] and of 'bicelles' by short-chain micelle forming phospholipids [79,80].

The fusion of liposomal vesicles that often occurs during the solubilization process has been observed to be correlated with an increased lipid fluidity and a decreased static order of the lipid hydrocarbon chains [206,207]. The phenomenon is related both to the nature of the detergent and of the lipid, and in particular the liposomal size [202]. With regard to the mechanism Edwards [81] has pointed out that, due to the wedge shape of detergent molecules, one might have anticipated a decrease, rather than an increase in vesicle size. However, it should be considered that small vesicles (< 30–50 nm) are under a considerable strain due to a high surface curvature. Small vesicles are thus farther removed from thermodynamic equilibrium than large vesicles, but on the other hand they are stabilized by kinetic barriers that efficiently prevent fusion in the absence of fusogenic

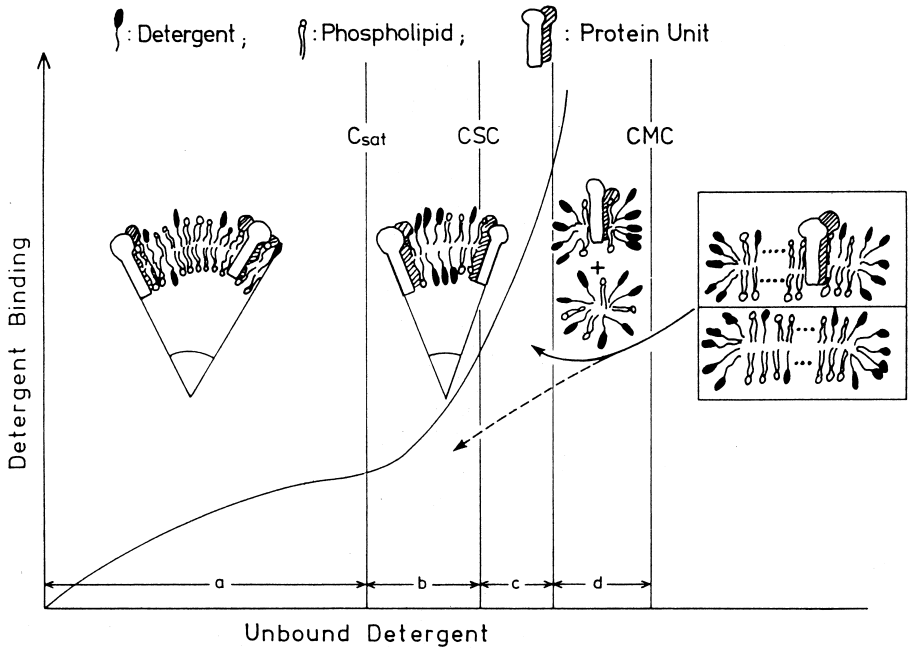

Fig. 2. Diagrammatic representation of the various phases encountered in the solubilization of protein containing membranes as a function of the free detergent concentration. In phase a, detergent is non-cooperatively taken up by the lipid phase; in phase b, above a free detergent concentration termed C_{sat}, detergent molecules cooperatively interact in the membrane and start to produce large membrane fragments which presumably are sealed at the edges by toroid assemblies of detergent molecules (see inset) but no solubilization of the vesicles occurs; in phase c, above a detergent concentration termed CSC (the critical solubilization concentration) lipid and protein containing units (monomers, protomers, oligomers) start to become solubilized as a variety of small, non-sedimenting objects such as very small membrane sheets or some type of mixed micelles; in phase d, only mixed lipid–detergent micelles and detergent solubilized protein units, covered by detergent and any remaining lipid, are present. Data taken from Kragh-Hansen et al. [66] with permission.

agents [212]. The presence of detergent may change this situation by facilitating the formation of transitory structures, leading to rapid fusion (over a time course of seconds or minutes instead of weeks or months) of vesicles with a thermodynamically stable state. In accordance with this view addition of detergents to preparations of multilamellar or large unilamellar vesicles ordinarily does not give rise to an increased light scattering [202,207].

The account given above leads to the following schematic picture of the solubilization of protein-containing membranes (Fig. 2): In the first stage detergent is taken up in non-micellar form, predominantly by the lipid phase. At some point this is followed by detergent-detergent interactions leading to destabilization of the bilayer structure and membrane fragmentation. Further detergent addition leads to the formation of mixed lipid–detergent micelles, exposure of the membrane proteins to micellar detergent, and protein solubilization. In practice, the transitions between these different forms are usually not sharp, but overlapping. The exact point of destabilization depends on the lipid species and heterogeneous mixtures therefore do not show first order transitions. In general, similar conclusions regarding the onset of detergent solubilization below the CMC have also been reached in other studies on lipid and protein systems [82–84]. Experimentally the different phases of detergent solubilization can be obtained by addition of graded amounts of detergent to reach a desired endpoint whose properties can then be studied by a number of physico-chemical and morphological techniques. However, as will be shown below, it is also possible in kinetic experiments to follow the time course of the various phases on the basis of light scattering changes observed after addition of fully solubilizing amounts of detergent.

Compared to many other biological membranes the Ca^{2+}-ATPase membranes used in these studies have a relatively simple phospholipid composition

and are characterized by the virtual absence of other membrane proteins than Ca^{2+}-ATPase. However, we believe that depending on the proper solubilization conditions the experience gained with the Ca^{2+}-ATPase and SR-lipid model systems have general significance for a comprehensive view of the detergent solubilization process, although both the protein and lipid heterogeneity in ordinary biological membranes adds more complexity to the issue. As a first approximation, solubilization of lipid and genuinely integral membrane proteins are almost concomitant processes, but subtle differences may lead to different profiles for the detergent extraction of the integral membrane proteins present in a given membrane as for instance in the solubilization of cytochromes from mitochondrial membranes [1]. Membrane proteins with strong protein–protein interactions such as junctional proteins cannot be solubilized by ordinary detergents. Protein–protein interactions in bacteriorhodopsin such as they occur in the membrane-bound patches in the halobacterial membrane are also hard to disrupt, but can be broken slowly (over a period of a day) by Triton X-100 [85].

Non-ionic detergents with long hydrocarbon chains are usually inefficient solubilizers of biological membranes. Other detergents have intermediary solubilizing prowess, e.g., cholate which does not solubilize bacteriorhodopsin [86] or Ca^{2+}-ATPase (unpublished observations) to a monomeric state, but is useful as a vehicle to obtain partial delipidation and incorporation of exogenous lipid in Ca^{2+}-ATPase membranes [69,87]. Some lipids like glycolipids together with cholesterol form separate and detergent-insoluble membrane 'rafts' with specific membrane proteins [88–90]. Cytoskeletal proteins ordinarily do not interact with non-ionic detergents, but by their interaction with cellular membranes may modify the solubilization of integral membrane proteins [91] or form complexes with membrane proteins after solubilization as has been observed for ankyrin and fodrin which bind to, e.g., Na^+,K^+-ATPase [92–94] and the erythrocyte anion exchanger [95].

2.3. *Role of flip-flop and extraction into pre-formed micelles*

From the experiments described in [66,67] it has been pointed out that for most detergents an essential condition for efficient solubilization is the onset of cooperative detergent–detergent interactions in the membrane lipid phase. The extent to which efficient solubilization occurs is likely to be affected by the degree with which detergents are able to penetrate and cross the membrane. Due to the hydrophilic–hydrophobic properties of the polyoxyethylene chains [96], detergents like $C_{12}E_8$ and Triton X-100 can be expected to flip-flop rapidly across the membrane, facilitating the formation of toroidal ring structures as shown in Fig. 3A. On the other hand, detergents with strongly hydrophilic heads can be expected to flip-flop at a slow rate, resulting in delayed solubilization. This is the case for dodecylmaltoside and dodecylsulfate which only slowly solubilize pure liposomal membranes [42,67,211,213]. In Stage I addition of dodecylmaltoside led to a slow and incomplete release of intravesicular carboxyfluorescein [213]. We consider it likely that in this situation dodecylmaltoside by interaction with the outer bilayer, functions as a fusogenic agent, similar to what has been reported for PEG induced fusion of small liposomes [212]. Furthermore, we have found that while $C_{12}E_8$ readily passes liposomal membranes [97], both dodecylmaltoside and SDS exhibit a very slow flip-flop rate [67]. Bile salt in pure liposomes ([98,99] and Schubert, quoted in [100]) and erythrocyte membranes [101] also apparently have very slow flip-flop rates which are critical for membrane destabilization. It might be that the solubilization which eventually does take place with dodecylmaltoside and SDS is caused by extraction of phospholipid molecules directly from the membrane into preformed detergent micelles (Fig. 3B). This may also be the mechanism behind the slow solubilization taking place when detergents with bulky head groups or long hydrocarbon chains like Tween-20 and Lubrol WX are used [42]. However, we have found that the rate of solubilization of SR vesicles by dodecylmaltoside is fast (within the second range [42]). Since SR vesicles, like pure lipid membranes, are impermeable to low concentrations of dodecylmaltoside [67], some additional factor(s) aiding in solubilization at high concentrations must be at work in this case. The most plausible explanation seems to be that the presence of protein in the membrane produces some disorder in the organization of lipid molecules which

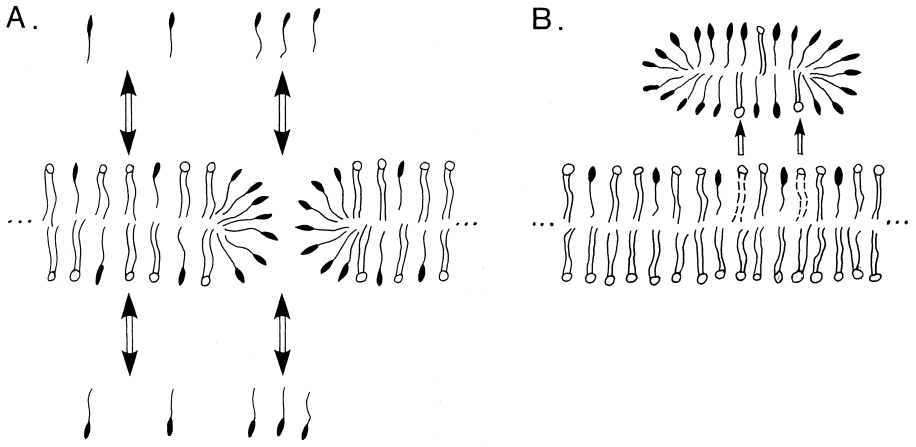

Fig. 3. Fragmentation and two modes of solubilization of lipid membranes by detergent. (A) Illustration of cooperatively binding assemblies of detergent molecules spanning the membrane. The figure illustrates the presence of non-micellar detergent on *both* sides of the membrane, resulting from rapid flip-flop and leading to the formation of toroidal detergent assemblies. (B) Extraction of phospholipid from the outer bilayer leaflet by direct transfer into micelles via the aqueous membrane water interface. This mechanism is suggested to account for slow solubilization of phospholipid observed with detergents with strongly hydrophilic heads (e.g., DM and SDS). Data taken from Kragh-Hansen et al. [67] with permission.

permits easier access of detergent to cooperatively interact with the Ca^{2+}-ATPase membrane than with the more orderly distributed lipid molecules in liposomes.

2.4. Solubilization by micellar attack

The question arises whether detergent solubilization of membranes generally proceeds via the uptake of non-micellar detergent into the membrane. We may consider that when we add a stock solution of detergent micelles with a low CMC to solubilize a membrane preparation, it is only the small fraction of non-micellar detergent present in the sample that is the active solubilizing species, while the detergent micelles function as a reservoir to continually replenish the pool of non-micellar detergent being removed because of the interaction with the membrane. However, in a number of situations we have observed that the transition from noncooperative to cooperative interaction in the membrane by non-micellar detergent proceeds at such a slow rate as to question if this is a realistic mode of solubilization. This is for example the case in equilibrium dialysis *trans* experiments where detergent is added to the compartment opposite to that of the dialysis cell containing the membrane preparation. In this experimental set-up the dialysis membrane will protect the membranes against exposure to micelles, yet it will permit the passage of non-micellar detergent above the critical concentration for cooperative interaction with detergent (cf. Fig. 1 of [66]). Yet, under *trans* conditions the establishment of equilibrium conditions for detergent binding and membrane solubilization is very slow, occurring on a time scale of weeks instead of 1.5–2 days as in *cis* experiments. The same kind of experimental situation can be arranged in gel equilibrium chromatography by exposing Ca^{2+}-ATPase membranes during their passage through the column to the continuous presence of detergent at a solubilizing concentration just below the CMC. In such experiments it is found for both $C_{12}E_8$ and DM (unpublished observation) that solubilization proceeds extremely slowly.

However, the experiments mentioned above are not entirely without problems, due to the restrictions in the supply of detergent for solubilization, imposed by the limited range of concentrations at which solubilization can proceed below the CMC. To address this issue more directly we have initiated a study of the kinetics of the solubilization process during exposure to detergent micelles. Assume first that solubilization only proceeds via non-micellar detergent. In this case we shall expect solubilization to occur at virtually the same rate at increasingly high detergent concentrations, due to the almost constant concen-

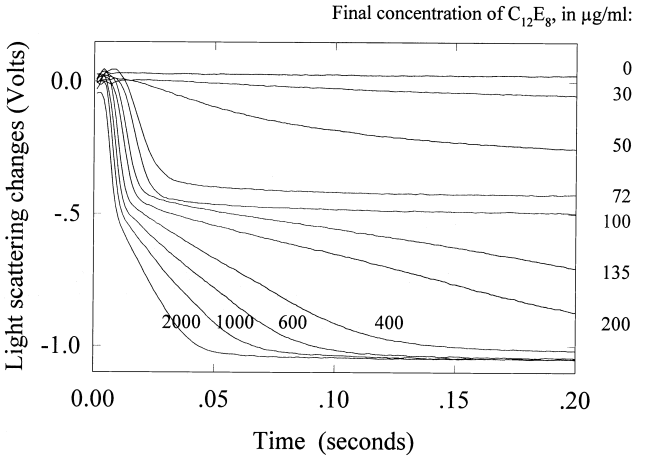

Fig. 4. Stopped-flow recordings of the changes in light scattering of SR vesicles produced by addition of $C_{12}E_8$ over a wide range of detergent concentrations. One volume of SR vesicles (at 0.2 mg protein/ml) was mixed with nine volumes of $C_{12}E_8$ (at a concentration 1.1 times the final concentration) in a Bio-Logic SFM-3 stopped-flow equipment. Changes in light scattering by the sample (expressed in volts) were observed at 90°, with an excitation wavelength of 312 nm (where the Hg-doped xenon lamp has a high-intensity output). The medium contained 100 mM KCl, 50 mM Tes–Tris (pH 7.5), 1 mM Mg^{2+} and 0.1 mM Ca^{2+}. Temperature was 20°C. The various traces have been shifted vertically by a few millivolts with respect to each other, for clarity. Control experiments (not shown) showed that the final level of light scattered by fully solubilized vesicles was hardly distinguishable from light scattered by buffer alone (Champeil et al., unpublished data).

tration of non-micellar detergent above the CMC. On the other hand, if detergent micelles represent the only solubilizing species no rate limitation is to be expected at high detergent concentrations. To see how these predictions work out, consider the kinetic data of Fig. 4 which show the changes in light scattering of SR vesicles produced by addition of $C_{12}E_8$ to SR vesicles over a wide range of detergent concentrations. As can be seen from the figure, traces obtained for $C_{12}E_8$ concentrations higher than the CMC are characterized by a lag phase during which light scattering remains quasi-constant, followed by an abrupt decline phase, virtually independent of the amount of detergent added. Subsequent to that there is a second decline phase whose decay rate depends significantly on the amount of detergent added. Tentatively, the lag phase can be identified with partitioning of detergent into the membrane up to a saturating level, which is followed by vesicle fragmentation (the first abrupt decline phase) and solubilization of lipid and protein (the second decline phase of light scattering). At fully solubilizing $C_{12}E_8$ concentrations (0.2–2.0 mg/ml) neither the lag phase nor the first decline is appreciably affected by changes in the detergent concentration. On the other hand, the second decline is strongly dependent on the total detergent concentration. These kinetic data support detergent micelles as active participants in the final stage of the solubilization process. In conclusion, it appears probable that the activation energy required for direct interaction with intact vesicles is too large for this to be a significant pathway during detergent solubilization of intact membranes, as previously suggested by Smit et al. [102]. On the other hand, after the formation of vesicle fragments detergent micelles can be envisaged to interact with these structures from the peripheral side by an edgewise approach to aid in the final phase of lipid and protein solubilization.

2.5. Solubilization of the protein component

The ability of detergents to extract integral membrane proteins from biological membranes is generally contingent upon their ability to solubilize membrane lipid. Concomitantly with the removal by detergent of a substantial part of the lipid, the hydrophobic membrane embedded sector of the membrane proteins becomes enwrapped in a layer of protective detergent coating. At this stage the membrane protein can be considered to be in a solubilized state, unless extensive protein–protein contacts prevent or retard dissociation of the protein units, as in the solubilization of bacteriorhodopsin [103,104]. Regardless of the existence of specific protein contacts removal of all, or virtually all, lipid is usually required to ensure solubilization at the protomeric level, to avoid non-specific hydrophobic contacts, mediated by lipids and gluing protein subunits together (cf. Fig. 7B for Ca^{2+}-ATPase and [105] for cytochrome oxidase). This may require treatment with excess detergent, e.g., by chromatographic procedures and sucrose density centrifugation [1,7]. On the other hand, if the objective is to maintain protein function in the detergent solubilized state it may be unwise to carry delipidation and deaggregation of oligomers too far, e.g., Na^+,K^+-ATPase [106,107] or the cytochrome b_6f complex [108] cannot with-

stand complete delipidation and deaggregation without loss of enzyme activity. On the other hand, it is a remarkable feature that most of those membrane proteins which have been successfully crystallized from the detergent-solubilized state can be completely, or nearly completely delipidated, e.g., reaction center [45], porin [110], cytochrome oxidase [111], and *Neurospora crassa* H^+-ATPase [112]. In some cases however some lipids had to remain associated to obtain crystals (specific lipids in the case of the plant light-harvesting complex [109], phosphatidylcholine in the case of the Ca^{2+}-ATPase [214]).

In the above description of the typical course of detergent solubilization we have presupposed the use of a 'mild' detergent which does not grossly affect the conformation of the protein after solubilization. Most non-ionic and bile salt (steroid-based) detergents or short-chain phospholipids fall within this category, but nevertheless it should be realized that solubilization in a quasi-native conformation does not automatically ensure retention of functional properties. For this, the choice of the correct detergent is a critical factor which must be established by trial and error. But also other medium conditions, including pH, ionic strength, the presence of lyotropic salts, protective ligands and chemical 'chaperones' such as glycerol and sucrose need to be considered [7,113–115]. Solubilization during detergent addition can be conveniently monitored by following the decrease in light scattering during stepwise addition of detergent. When light scattering reaches a minimum, non-solubilized (very often inactive and aggregated or cytoskeletal) proteins can be removed by centrifugation at medium speed (e.g., for 60 min at $105\,000 \times g$; if higher speeds are used, a particular point to take into consideration is that labile proteins may become inactivated by the increase in hydrostatic pressure resulting from centrifugation, as in their membrane-bound state ([116]; Esmann, personal communication)).

The physicochemical properties of a number of detergents which may be considered for solubilization with retention of functional properties were already shown in Tables 1 and 2. From our own experience we draw attention to the following points. It appears that the right choice of detergent for solubilization is often a question of a balance between 'mildness' and efficiency in covering the hydrophobic surfaces of the membrane sector. Inefficiency of interaction with the protein may be the reason why we generally find that C_{16}–C_{18} detergents are unsuitable for solubilization and retention of functional properties, at least for delipidated proteins, despite their greater similarity to membrane lipids [43]. For many unstable proteins such as Ca^{2+}-ATPase [42,43], Na^+,K^+-ATPase [117], and cytochrome oxidase [26,118] non-ionic detergents of an intermediary size like $C_{12}E_8$ and dodecylmaltoside are optimal. Triton detergents (Triton X-100, Nonidet) may be used with retention of activity in a number of cases, but in general these compounds do not appear to be superior to pure hydrocarbon detergents. Among the steroid-based detergents CHAPS appears as a good candidate by combining efficiency of solubilization with stability in the detergent solubilized state in particular for receptors (see, e.g., [119]). However, the rigidity of the steroid nucleus often prevents expression of enzyme activity in the detergent solubilized state. More abrasive detergents, like octylglucoside, C_8E_5, and DDAO are very efficient solubilizers, but require a robust protein structure to avoid inactivation. On the other hand, they are good candidates for delipidation and crystallization, if the membrane protein can withstand their deteriorating effect.

3. Modes of detergent–membrane protein interaction

3.1. The detergent solubilized state

The exact way by which detergents interact with membrane proteins has been the subject of extensive speculation. The unique properties of 'mild' detergents in their interaction with membrane proteins is indicated by the fact that these compounds do not interact to any noticeable degree with most water-soluble proteins, except those which like serum albumin can accommodate small amounts of detergents inside their hydrophobic pockets. For membrane proteins it was originally proposed that the large hydrophobic sector of integral membrane proteins becomes surrounded by a detergent micellar-like oblate structure as shown schematically in Fig. 5A [2,15] This can be envisioned to occur either by insertion of the membrane protein into a preformed detergent micelle (Fig. 5D) at detergent concentra-

tions above the CMC, as suggested by Kleinschmidt et al. [120]; alternatively, the hydrophobic sector might act as a nucleus for the formation of a surrounding micellar structure at detergent concentrations below the CMC. In favor of a micellar mode of interaction it has originally been pointed out that the amount of detergent bound by various membrane proteins like cytochrome b_5 [121], bacteriorhodopsin [122] and glycophorin [123], is similar to the number of detergent molecules present in pure micelles. However, model calculations indicate that the appreciable size of the inserted membrane protein will impose a considerable increase in the amount of bound detergent as compared to the size of a micelle, if micellar characteristics such as their typical dimensions and surface area per detergent monomer are to be retained [17,124].

As an alternative we have previously proposed [17,124], as shown in Fig. 5B, that detergent covers the hydrophobic protein surface with a monolayer, similar to the arrangement of detergent molecules at an air–water interface (Fig. 5E). This mode of interaction leads to a more economical detergent binding, since in contrast to micellar binding all detergent molecules can be considered to be in contact with the binding protein. In accordance with this concept we find that detergent binding of various detergents to a given membrane protein (bacteriorhodopsin, reaction center, SR Ca^{2+}-ATPase, and cytochrome oxidase) is inversely correlated with the cross-sectional area of these detergents, determined at an air–water interface [17]. However, it is clear that, due to the curved and irregular structure of the transmembrane segment, description of detergent binding in terms of a monolayer structure can only be an approximation. In addition it may be argued that to avoid the hydrophilic–hydrophobic contacts between water and detergent molecules at the ends of the transmembrane belt as depicted in the monolayer model shown in Fig. 5B, these detergent molecules need to be placed

Table 3
Estimated average cross-sectional areas (CSA) and hydrophobic chain lengths (h_D) of detergent molecules bound as prolate monolayer rings around selected membrane proteins or bound at an air–water interface

Detergent	Bacteriorhodopsin		Reaction center		Ca^{2+}-ATPase		Cytochrome oxidase		Air–water	
	CSA (nm^2)	h_D (nm)	CSA (nm^2)	h_D (nm)	CSA (nm^2)	h_D (nm)	CSA (nm^2)	h_D (nm)	CSA (nm^2)	h_D (nm)
$C_{12}E_6$	0.51	0.64	0.63	0.52	0.79	0.41	0.77	0.42	0.57	0.57
Triton X-100	0.49	0.73	0.78	0.46	0.90	0.40	0.80	0.45	0.57	0.63
DM	0.31	1.06	0.55	0.59	0.45	0.72	0.56	0.58	0.46	0.71
DDAO	–	–	0.30	1.10	0.30	1.08	0.37	0.87	0.38	0.86

The calculations are based, on the one hand, on the measured detergent binding capacities of the membrane proteins in their fully detergent solubilized protomeric forms, summarized in Table III of Møller and le Maire [17], and, on the other hand, on the dimensions of the protein hydrophobic surfaces that can be estimated from the published structures.

Average cross-sectional areas (CSA) and average lengths of the hydrocarbon chain (h_D) for bound detergent were estimated by appropriate modification of a derivation, shown as Eq. (A3) in Møller and le Maire [17]:

$$V_H(\text{prol}) = 1/2\pi H h_D p + 2/3\pi H h_D^2$$

where V_H(prol) is the total hydrophobic volume of bound detergents, p and H are the perimeter and the height of the hydrophobic transmembrane sector of the protein of interest, respectively. The following values were used for the dimensions of the membrane proteins based on the structural data: bacteriorhodopsin (including tightly bound lipids): $p = 12$ nm, $H = 3.0$ nm, [126]; reaction center: $p = 16.5$ nm, $H = 3.0$ nm, [45]; SR Ca^{2+}-ATPase: $p = 17$ nm, $H = 2.5$ nm, [125]; cytochrome oxidase: $p = 25$ nm, $H = 3.0$ nm, [111]. Starting from the number n of bound detergent molecules (Table III of Møller and le Maire [17]), V_H(prol) was calculated from the formula $V_H(\text{prol}) = n\bar{v}_D M_D/N_{av}$ where N_{av} is Avogadro's number, M_D is the molecular mass of the hydrocarbon chain and $\bar{v}_D$ the partial specific volume of the hydrocarbon chain (taken as 1.155 and 1.150 cm^3/g for C_{12}-detergent and Triton X-100, respectively). Then, using the estimated perimeter and the height of the hydrophobic transmembrane sector of the protein and using the equation above, we estimated h_D for bound detergents. Finally, CSA is defined by the fact that the product (CSA × h_D) represents the hydrophobic volume of each detergent molecule; this volume was calculated as $\bar{v}_D M_D/N_{av}$. The values for CSA and h_D at an air–water interface, shown in the last column of the table, are based on estimates of the CSA summarized in Table I of Møller and le Maire [17]. In this case, h_D was calculated from $h_D \times \text{CSA} = \bar{v}_D M_D/N_{av}$.

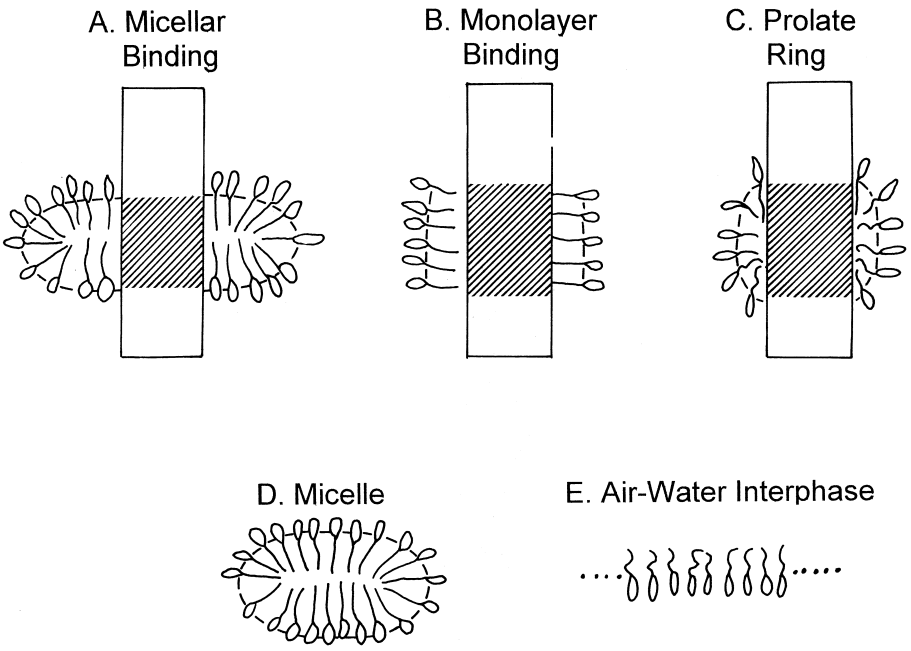

Fig. 5. Models of detergent binding by membrane proteins. (A) Micellar type of arrangement. The membraneous sector (hatched area) of a membrane protein is embedded in an oblate detergent ring with approximately the same characteristic dimensions as those of a pure micelle [2], i.e. with the same semi-axis of the hydrophobic core, e.g., for a $C_{12}E_8$ micelle, about 1.2, 2.8, 2.8 nm [15]. (B) Pure monolayer type of arrangement. The hydrophobic region is covered by a layer of contiguous detergent molecules in contact with the membrane protein, leaving detergent hydrophobic regions exposed at the edges. (C) Prolate monolayer ring arrangement. Note that both the membrane protein hydrophobic surface area and the hydrophobic moiety of detergent molecules are shielded from contact with the aqueous medium in this arrangement. Previous similar covering of transmembrane sector have been proposed (see Fig. 2B of [124], Fig. 6B of [17] and Fig. 1A of [154]). (D) Structure, for comparison, of an oblate micelle of pure detergent [2,15]. (E) Disposition of detergent molecules at an air–water interface.

closer to, and parallel to the transmembrane sector. This means that only the detergent molecules covering the middle of the transmembrane segment can have a monolayer arrangement relative to the transmembrane segment, while the direction of the hydrocarbon chains at both ends will be parallel to the transmembrane segment as shown in Fig. 5C (see also [17,124,154]). This will produce a curvature for detergent bound consistent with the 'wedge' shape of detergent molecules and will result in the formation of what we may term a prolate monolayer ring around the hydrophobic membrane sector, and similar to what Haneskog et al. have termed a semi-elliptical torus [154]. Using this model, and starting from reasonable estimates of the hydrophobic surface areas of the membrane proteins (based on the available structures from two-dimensional (2-D) or 3-D crystals [45,111,125,126]) combined with the measured number of bound detergent molecules, (based on determination of the binding capacity of various membrane proteins [17]), we present in Table 3 calculated values for the dimensions (cross-sectional areas and lengths of the hydrocarbon chains) of the individual detergent molecules bound. These calculations indicate that there are systematic differences between the cross-sectional areas and lengths of the hydrocarbon chains of the different types of bound detergents, in the same direction as at an air–water interface (such that $C_{12}E_8 \sim$ Triton X-100 $<$ dodecylmaltoside $<$ DDAO for hydrocarbon chain lengths, and vice versa for the cross-sectional areas, since the product ($h_D \times$ CSA) represents the volume of the hydrocarbon chains of the detergents, cf. the legend to Table 3). Furthermore, there are no systematic differences between cross-sectional areas and hydrophobic chain lengths for detergent bound to a membrane protein versus that of a monolayer in equilibrium with micelles at an air–water interface, despite that there must be significant differences between the two situations (in particular the presence

of surface curvatures and van der Waals interactions in the binding of detergent by membrane proteins). With the exception of DDAO, the hydrophobic chain lengths are in all cases considerably smaller than they are expected to be in their extended state or in the hydrophobic core of a micelle (1.1-1.2 nm, see [15]). The calculations thus suggest the hydrophobic sector of the membrane proteins to be covered with a rather dense layer of detergent having physicochemical properties that may be quite different from the fluid, dynamic state characteristic of detergent micelles.

It can also be seen from Table 3 that the cross-sectional areas for binding of a given detergent to reaction center, SR Ca^{2+}-ATPase or cytochrome oxidase are estimated to be quite similar, suggesting that, for *large* membrane proteins, binding measurements of this detergent can be used as a semiquantitative measure of the hydrophobic surface area of an unknown membrane protein. On the other hand, the cross-sectional area of detergents bound to bacteriorhodopsin is calculated to be smaller, indicating a higher degree of detergent binding per unit hydrophobic surface area than for the three other membrane proteins. Possibly, bacteriorhodopsin being an almost completely membrane embedded membrane protein can be enwrapped more fully with detergent than larger membrane proteins like, e.g., SR Ca^{2+}-ATPase where the projecting cytosolic polypeptide mass [125] may cause steric hindrance. In the case of the 3-D crystal of reaction center from *Rhodopseudomonas viridis* studied by neutron diffraction with contrast variation (see below, Section 3.2) there are indications that detergent binding can be locally limited by hydrophilic regions (near helix H, see Fig. 1a in [143]). For two other bacterial membrane proteins, Fhua, a 22 antiparallel β-strand porin like protein [127] and LacS, with 12 predicted transmembrane helices (Poolman et al., unpublished observation), the number of DM molecules was also found to be high (about 200 molecules per monomer). In the case of Fhua the structure was not known at the time when the detergent binding measurements were done, but we predicted from our binding data a perimeter of the protein in the range of 12–18 nm [127]. When the structure came out the perimeter of the protein was found to be about 16 nm [128].

3.2. Information on detergent binding deduced from 3-D crystals of membrane proteins

In 1980 for the first time two membrane proteins were crystallized from a detergent solution, bacteriorhodopsin [129] and porin [130]. About 30 well-diffracting 3-D crystals of membrane proteins have been now reported and the use of detergents for this purpose are dealt with in a number of recent reviews [63,131–133]. It appears that, so far, short-chain detergents have been generally more successful than longer ones for crystallization, maybe because they fit more easily around the hydrophobic regions of the proteins without hindering hydrophilic inter-protein contacts: for example in Table 3 of [63], out of 34 crystallization conditions for membrane proteins, 21 use C_8 detergents and only six use C_{12} detergents; however, recently, several successful crystallizations based on the use of dodecylmaltoside have been reported. This detergent, as mentioned above, is generally less denaturing than octylglucoside for fragile enzymes (see, e.g., the mechanosensitive ion channel, crystallized in dodecylmaltoside [134], and the fumarate reductase, crystallized with a mixture of dodecylmaltoside and decylmaltoside [135]). In systematic studies of crystallization in different detergents C_{11} or C_{12} detergents were found to be as successful [59] or even more successful [57] than shorter ones. Note that Vinogradova et al. [136] reached also the conclusion that medium chain detergents are generally preferred for use in NMR studies of membrane proteins because they are no worse than short-chain detergents (C_8) in terms of increasing the effective molecular mass of the protein–detergent complex of interest while they are considerably better at maintaining a native-like protein conformation. The first atomic structure of a membrane protein, a photosynthetic reaction center, was described in 1985 [45] and now more than 20 structures from a dozen families of proteins are resolved with a resolution of 3.5 Å or better [137]. Most of these structures come from 3-D crystals but a few are based on 2-D crystals. In the latter case, detergents are generally removed from the protein and replaced by lipids (however, see [138]). Specific reviews exist also for this type of crystallization (e.g., [139] and Rigaud et al., in this issue). Finally it is of interest to mention that lipidic cubic phase mediated crystallization of

membrane proteins was demonstrated by growing bacteriorhodopsin microcrystals in a monoolein cubic phase [140,141]. The recent development of this technique shows that, at least in the case of the purple membrane, detergent solubilization is not required to obtain these crystals [142].

In the case of classical 3-D crystals of membrane proteins, the entity which crystallizes is a protein–detergent complex, sometimes including residual lipids. However, only a few of the bound detergent molecules, if any, are sufficiently well ordered to be seen in the electron density maps. All the other molecules of detergent (sometimes several hundred per protein molecule) are disordered, maybe because of dynamic exchange with the environment. In four crystals the structure of the detergent phase has been studied by low-resolution (1.3–1.6 nm) neutron diffraction: in the reaction center from *R. viridis* in DDAO [143], in the reaction center from *R. sphaeroides* in octyl glucoside [144], in the tetragonal crystals of OmpF porin trimers in β-OG and in C_{10}DAO; [145]) and in the trigonal crystal of OmpF porin trimers in *n*-octyl-2-hydroxyethylsulfoxide/C_8E_{10} [146]. This is a small number of studies, considering the number of crystals now available, but these very interesting experiments are difficult to perform in part because they require stable crystals of large size, (ideally 0.5–1 mm^3) and such a high flux of neutrons that only one source for these neutrons is available (Institut Laue-Langevin at Grenoble). Models of the structure of detergent phase in the crystal are obtained by contrast variation (by varying the H_2O/D_2O ratio in the solvent) and by using the atomic coordinates determined by X-ray diffraction. Remarkably, except in the case of the trigonal crystal of porin, the detergent forms a continuous belt around the proteins, the size of which is about 1.5–2.0 nm in the direction normal to the α-helices or the β-sheets of the transmembrane sector and 2.5–3.0 nm parallel to it. This latter value corresponds to the thickness of the hydrophobic part of the bilayer. When two different detergents were used for homologous proteins (reaction centers: β-OG and DDAO) or for the same protein (tetragonal crystal of porin: β-OG and C_{10}DAO) the data showed quasi-identity of position and shape of the detergent rings around the proteins ([144,145] see Fig. 6A–C). Since the molecular length of these detergents, including the polar head, is about 1.5 nm the thickness of these rings (Fig. 6C) is consistent with a prolate monolayer ring around the hydrophobic membrane sector, as discussed above in Section 3.1 (cf. Fig. 5C). Furthermore, for one of these crystals (porin in C_{10}DAO; [145]) the electron density map, obtained at a D_2O content such that the detergent tails are contrast matched, showed not only the protein density but also density at about 1.5–1.8 nm from the trimer surface which is likely to correspond to the amine oxide head groups. This 'outer ring' feature was also observed in the case of the 2-D crystal of the H^+-ATPase obtained from a DM-solubilized preparation of this protein and studied by electron crystallography [138]. In the trigonal crystal of porin, the belt, which has about the same size as described above, appears discontinuous: with this different crystal packing, some protein-protein contacts between trimers of porins seem to prevent the formation of a continuous toroidal annulus [146].

Another interesting feature of the neutron-scattering studies concerns the type of amino acid in contact with the detergent layer. The area to which the detergents bind contains almost exclusively aliphatic residues, and this zone corresponds to the hydrophobic part of the bilayer where the acyl chains of lipids are present. This emphasizes again the fact that, in protein–detergent complexes, detergent covers the regions normally covered by lipids, as was deduced many years ago on the basis of indirect evidence [1,2]. In addition, the lower and upper boundaries of this area often contain two bands of aromatic residues, tyrosines pointing away from the detergent belt and interacting with the polar head groups and phenylalanines pointing inwards, towards the tail of the detergent monomers. This feature has been noted in particular for the porin crystals [145,146]. Interestingly, in the recent 3-D structure of bacteriorhodopsin at 1.9 Å resolution nine lipids per monomer (out of a total of ten) could be modeled in the electron density map and five tyrosine and two tryptophan residues were found to be located in the vicinity of lipid ethers or head groups [126].

In addition to binding to the hydrophobic part of proteins in the crystal, the ribbon-like detergent structure may be interconnected with its neighbors by bridges of fused detergent layers. This is particularly the case for reaction center [144]. However, this

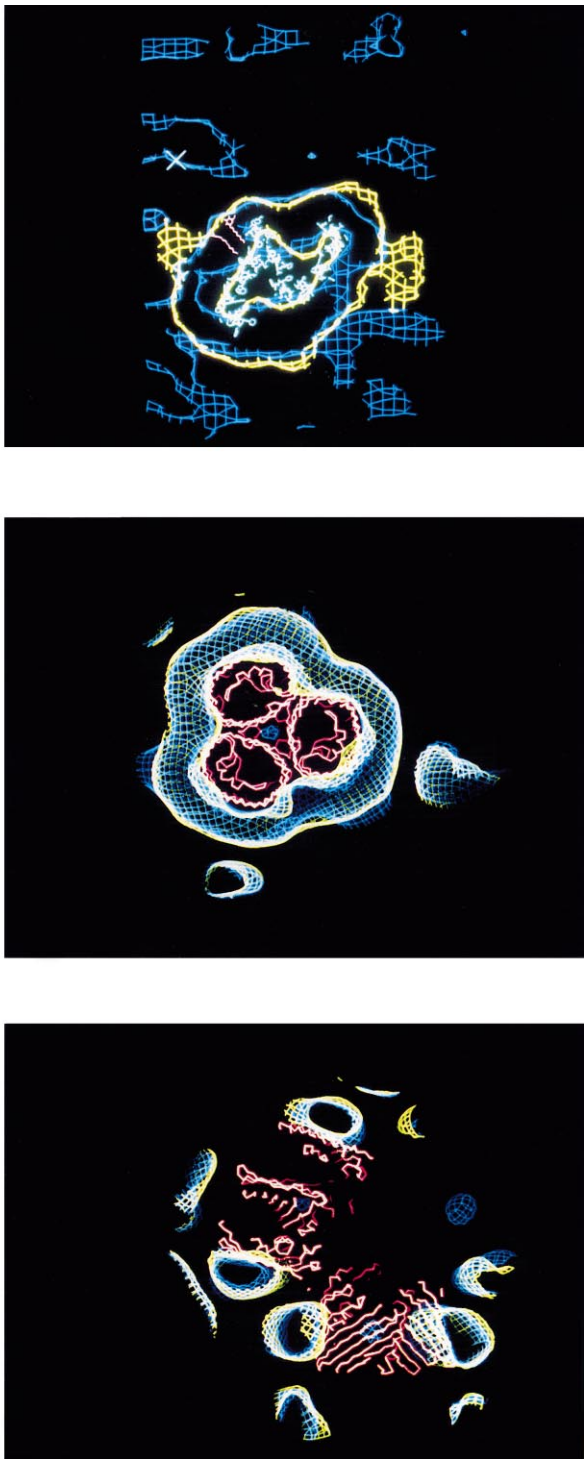

Fig. 6. Detergents rings around two types of membrane proteins as obtained by single-crystal neutron diffraction studies. (A) superposition of the contours of the detergent ring around the reaction centers from *R. viridis* (yellow) in DDAO [143] and from *R. sphaeroides* (blue) in β-OG [144]. These proteins are crossing the membrane with α-helical segments roughly perpendicular to the plane of the figure; the contours shown correspond to a position near the center of the bilayer. Inside the two contours, the authors [144] have arbitrarily placed two detergents molecules (in pink), with their polar head on the solvent side of the detergent phase and their hydrophobic tail pointing toward the hydrophobic transmembrane core of the reaction center molecules. It can easily be seen that the stretched length of both the β-OG and DDAO (about 1.5 nm) is nearly equal to the detergent ring thickness in the equatorial plane. Note that the size of pure DDAO micelles has been estimated using small angle X-ray scattering [32] or neutron scattering [147]. Assuming micelles are oblate ellipsoids [15], values for the semi-axis of 1.29, 2.76, 2.76 nm have been calculated [32]. (B,C) Two orthogonal views of the *E. coli* OmpF porin trimer, whose transmembrane sector is formed by β-barrels (see in red the α-carbon skeleton superimposed on the map), also in two different detergents: β-OG (green), and in C_{10}DAO (blue), in tetragonal crystals [145]. Under the conditions of this experiment, only the detergent hydrophobic core of the detergent rings is well visualized while the detergent head group is not well resolved. In B the porin trimer is viewed down its 3-fold axis. In both types of membrane proteins the modeled detergents belts superimpose almost exactly. The scales are nearly the same for the three photographs and in A the bars of the white cross have a length of 1.3 nm. Photographs are courtesy of Drs M. Roth and P. Timmins (IBS and ILL, Grenoble).

feature is not always present as it depends on the type of crystal packing: for example in the case of the trigonal crystal of porin, the detergent belts are individuals and surround solvent-filled columns traversing the crystal [146]; in the tetragonal crystals of porin, fusion of detergent layers as evidenced by continuous hydrophobic domains does not seem to play a significant role in the crystallization [145].

3.3. *A case for micellar binding by small membrane proteins?*

In a pioneering study on the binding of detergent to membrane proteins by Robinson and Tanford [121] it was shown that binding of Triton X-100, deoxycholate, and SDS by cytochrome b_5 is characterized by marked cooperativity, taking place close to the CMC and resulting in the binding of micellar amounts of detergent. This contrasts with the formation of a detergent monolayer at an air–water interface where there is a continuous build-up over the whole range of detergent concentrations in the bulk solvent phase from zero to the CMC. Cytochrome

b_5, with a molecular mass of 16 kDa, consists of a heme-containing cytosolic domain and membrane-inserted hydrophobic anchor. It could be shown that only the latter interacted with Triton X-100 and deoxycholate. Although both domains, as expected, interacted with SDS, the interaction of the membrane inserted sector with SDS was intriguing in the sense that it had different characteristics than observed for the unfolding of water-soluble proteins. The latter process usually takes place well below the CMC, presumably as the result of combined hydrophobic and hydrophilic interactions which leads to the formation of detergent clusters or micelle like structures below the CMC [148–150]. On the other hand, the interaction of SDS with the cytochrome b_5 membrane anchor, was characterized by binding of larger amounts of detergent than water-soluble proteins and this binding process took place close to the CMC. This could be the result of uptake of the transmembrane segment by an SDS micelle, justifying the current use of SDS to provide a membrane like environment for studies of the conformation of transmembrane segments by, e.g., NMR spectroscopy [50,151,152,200].

This raises the possibility that a micellar binding mode (Fig. 5A) might well be a realistic alternative to monolayer binding of detergents by membrane proteins with small membrane inserted sectors. However, the interpretation of binding data close to the CMC are subject to the caveat that the effect of accompanying changes of the protein need to be taken into account. In the above study cytochrome b_5 was in a delipidated and water-soluble, but aggregated form. In studies on a related NADPH–cytochrome P450 reductase it has been found by analytical ultra centrifugation measurements that addition of detergent close to the CMC results in the transition of the protein from an approximately heptameric to a monomeric state [153]. Evidently, keeping these membrane proteins in solution in the absence of detergent requires shielding of hydrophobic surfaces by oligomerization. Subsequent deaggregation with the ensuing exposure of these surfaces can then be expected to be accompanied by cooperative detergent binding at the CMC.

Concerning the detergent binding mode of small membrane proteins we therefore have to rely on data obtained above the CMC in their fully detergent solubilized, protomeric state. Few relevant studies have been published on this subject, mainly on single transmembrane fragments of fully or partially membrane inserted polypeptides. Lauterwein et al. [36], studying the interaction of melittin from bee venom with different detergents by a broad range of physicochemical methods concluded that the conformation of the polypeptide after interaction with detergent micelles is similar to that in a phospholipid bilayer environment, but that somewhat less detergent was bound than is present in pure micelles. Beswick et al. [51] examined the behavior of a hydrophobic peptide derived from the proteolipid of *Saccharomyces cerevisiae* and an amphipathic peptide from annexin. On the basis of NMR relaxation experiments they obtained evidence that the uptake of these peptides in DPC micelles did not affect micelle dynamics, but resulted in some ($\sim$20%) increase of micelle size. In studies on putative transmembrane peptides derived from the C-terminal part of SR Ca^{2+}-ATPase (M6 and M7) we found by size exclusion chromatography that M6 in the presence of DM and SDS eluted at the same position as the pure micelles [53]. Furthermore, NMR and CD analysis disclosed that in the absence of detergent one of peptides (M6) was present as a random coil that forms small water-soluble aggregates, whereas, by the addition of detergent above the CMC, the peptide acquired secondary structure, suggestive of a native conformation [50,53]. In fact, by NMR, we made the unexpected finding that M6 adopts an helical structure only in its N-terminal part on 12 residues while the addition of TFE was required to reveal the propensity of the C-terminal segment to form also an helix. The two helical segments were linked by a flexible hinge region containing two important residues for calcium binding [50]. This unusual feature was later confirmed by high-resolution 3-D structure of the complete Ca^{2+}-ATPase [214].

In conclusion, from these studies it is apparent that detergent micelles may be considered to provide membrane-like environments around transmembrane peptides. However, more studies will be needed to substantiate this conclusion and the extension of the concept to whole membrane proteins with small membrane-inserted anchors.

3.4. Protection of membrane proteins by amphipols

Most membrane proteins (unlike the cytochrome proteins considered in the preceding paragraph) are very dependent on protection of their hydrophobic surfaces to prevent them from being irreversibly inactivated as a consequence of denaturation and inactivation. In 1996, a new class of amphipathic polymers, dubbed 'amphipol' polymers, was introduced as an alternative to classical detergents to keep membrane proteins in a solubilized state without denaturation. Amphipols consist of a polyacrylate backbone partially derivatized with octyl and isopropyl chains. Initial studies with four membrane proteins showed that complexes of these proteins with amphipols could indeed be handled in detergent free solution as if they were water-soluble proteins [155–157]. Recently, we evaluated the properties of amphipol A8-35 for use with SR Ca^{2+}-ATPase [158]. We found that the polymer was incapable of solubilizing the lipid of Ca^{2+}-ATPase containing membranes, but that after detergent solubilization the polymer protected Ca^{2+}-ATPase against aggregation and irreversible inactivation in a variety of situations. Activity in the presence of amphipol alone was low, but the enzyme could become reactivated by the addition of detergent. It might be that amphipol by a multipoint attachment stabilizes the hydrophobic surface area, but that the coverage is incomplete and therefore addition of detergent is required for full activity. Nevertheless, our observations suggest that amphipols added to detergents will provide useful tools for handling solubilized Ca^{2+}-ATPase, and presumably also other membrane proteins, under conditions that would otherwise lead to its irreversible denaturation and/or aggregation.

4. Structural investigation of detergent-solubilized membrane proteins

4.1. Size exclusion chromatography

A simple way to analyze the membrane components after detergent solubilization is by HPLC or FPLC, using silica gel or Superose columns. If the membrane protein sample is reasonably pure the chromatogram may readily reveal the self-associated

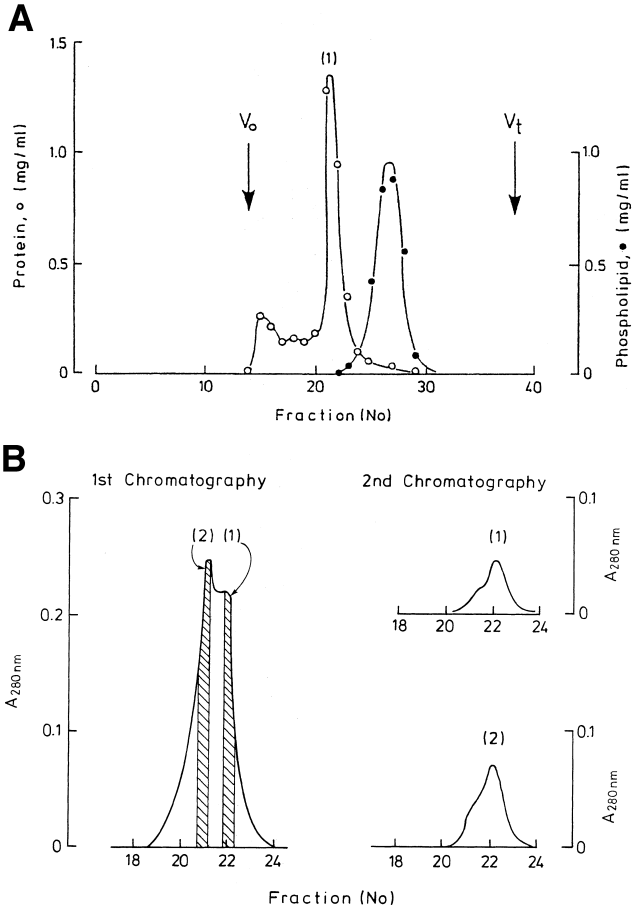

Fig. 7. Preparation of delipidated, monomeric Ca^{2+}-ATPase by HPLC on silica gel. Ca^{2+}-ATPase membranes (5 mg protein), prepared from sarcoplasmic reticulum, was solubilized with 50 mg dodecylmaltoside (DM), and after airfuging applied to a 0.75×60 cm SW3000 TSK column, equilibrated and eluted with DM (1 mg/ml), 20 mM Tes (pH 7.0), 100 mM NaCl, and 0.5 mM $CaCl_2$. Notice the presence of the predominantly monomeric Ca^{2+}-ATPase peak (1), which is separated from both phospholipid (●) and oligomeric or aggregated Ca^{2+}-ATPase, closer to the void volume, V_0. (B) Demonstration of reversible oligomerization of Ca^{2+}-ATPase monomer after partial relipidation. An aliquot of the monomeric peak fraction of the preceding experiment (approx. 0.6 mg protein) was treated with 0.35 mg dioleoylphosphatidylcholine (DOPC) and 0.40 mg DM and then applied to a Superose 6 (Pharmacia) column, equilibrated and eluted with DOPC (0.2 mg/ml), DM (1 mg/ml), 20 mM Tes (pH 7.0), 100 mM NaCl, and 0.5 mM $CaCl_2$. Notice that the presence of lipid resulted in partial conversion of monomer (1) to dimer (2), eluting as an extra peak. Rechromatograpy of Ca^{2+}-ATPase at monomeric and dimeric positions (shaded areas) also resulted in the appearance of a biphasic peak, but which this time was dominated by monomer. These findings are consistent with partial and reversible formation of dimeric Ca^{2+}-ATPase from monomeric Ca^{2+}-ATPase by lipidation.

state of the protein in terms of monomers, dimers, and higher oligomers. Usually monomeric protein elutes just ahead of the peak of mixed micelles of phospholipid and detergent (Fig. 7A). By comparison with water-soluble protein standards the Stokes radius of the protein–detergent complex can be estimated [162,163]. However, it should be taken into account that bound detergent by itself may make a significant contribution to the size of the complex. In addition, for polyethyleneoxide detergents the overall Stokes radius of the protein–detergent complex may be overestimated to some extent, probably due to different elution characteristics of the flexible polyethyleneoxide moiety as compared to that of the more compact protein polypeptide chain [16]. With DM on the other hand, the Stokes radii appear to be correctly estimated [127,158] and combined with measurements of the sedimentation coefficient, allow an alternative estimation of the molecular mass of the complex [7]. Detergent binding can be estimated by gel equilibrium chromatography with the aid of radioactively labeled detergent, as an increment in detergent concentration associated with the protein peak. For such estimations it should be taken into account that silica and Superose gels are not truly inert materials, but that they interact with monomeric (non-micellar) detergent [17]. Therefore the columns should be carefully equilibrated with detergent before application of the protein sample to saturate the column binding sites. Consideration should also be given to the presence of lipid released from the protein which forms mixed micelles with detergent. This usually causes a considerable rise in detergent concentration above baseline which often overlaps with the much smaller rise in detergent concentration pertaining to the protein peak. To avoid this complication it may be advisable to remove lipid before gel chromatography, e.g., by chromatography on DEAE–cellulose columns. Alternatively, one may estimate binding after rechromatography of an aliquot of the protein peak from the first gel chromatography. By rechromatography it is also possible to examine the reversibility of oligomer formation, see Fig. 7B and, e.g., [115]. If enough material is available reversible equilibria can be studied quantitatively by the use of large zone chromatography (e.g., [164]).

Comparing the performance of silica gel and Superose columns, the following generalizations can be made: silica gels usually give the best resolution of the membrane components; in particular we find that the peak of mixed lipid micelles is better separated from the protein monomeric peak. On the other hand Superose columns can be used over a wider pH range, especially at alkaline pH, and are less liable to deteriorate. Column deterioration is a cumbersome problem with the expensive silica gel columns, especially in the presence of detergent. To counteract column deterioration of silica gel columns we immediately remove detergent after each day of experiment, we periodically cleanse the column with first 6 M Gu.HCl and then with 20% methanol (sometimes also a third treatment with SDS) and we exchange guard columns at suitable intervals. Insoluble residues that are trapped may be removed by reversing the flow through the columns cautiously.

4.2. *Determination of the molecular mass of membrane proteins*

Analytical ultracentrifugation is the classical way of determination of molecular mass, but has somewhat got out of fashion. Nowadays it is more common to estimate molecular mass by SDS gel electrophoresis. Molecular mass of polypeptide chains can also be deduced from cDNA sequences, but one should be aware of errors that may be introduced by posttranslational modifications such as glycosylation. The use of SDS–PAGE may also lead to biased results, in particular with membrane proteins whose behavior deviates from that of the water-soluble standard proteins used for calibration. As for the M_r deduced from cDNA the molecular mass of a subunit or monomer is measured, but there are examples of retention of oligomeric structure of membrane proteins after SDS solubilization, also under reducing conditions to disrupt disulfide bridges (e.g.[165]).

Mass spectrometry is a technique that will find increasing use in the future. The challenge, however, is much greater for membrane proteins and hydrophobic peptides than for water-soluble proteins (see Barnidge et al. [166] for a recent and well-documented account of the situation). Thus matrix-assisted UV-laser desorption ionization mass spectrometry

(MALDI) has provided the correct monomeric and sometimes oligomeric M_r of several membrane proteins (e.g., monomeric porin from *Rhodobacter capsulatus* (M_r was correct within 0.3% [167]), trimeric and monomeric porin from *Escherichia coli*, [168]). This technique is also capable of providing information on subunit composition and can be used in the presence of detergents (e.g., [168,169]), but detergents lower the quality of the spectra and, in general, efforts are made to remove them before the measurements (e.g., [170]). Electrospray ionization (ESI), which can provide more accurate mass measurements, ($\pm$0.01–0.05% of the calculated theoretical mass), is even less tolerant to the presence of detergents than MALDI, and procedures have been developed to remove detergents before injection of the samples in the mass spectrometer: e.g., through precipitation by cold acetone [171–173] or chloroform/methanol [174], or through extraction into a non-polar solvent phase [166]. Promising approaches include the direct coupling with the electrospray of an additional chromatographic separation (HPLC [173,174]; fused-silica capillary [166]). The interest of mass spectrometry lies in its sensitivity and accuracy, so that post-translational modifications can be identified. However mass spectrometry is usually not able to provide information on the stoichiometry between subunits of oligomeric membrane proteins in their native membrane or after solubilization. Therefore analytical ultracentrifugation remains a method of choice for determination of M_r of membrane protein solubilized with a non-denaturing detergent; from the latter measurements the number of subunits in the native complex can be deduced if the M_r of the monomer and the detergent binding ratio (cf. above) are also known. Even if the detergent binding ratio is unknown the M_r of the solubilized protein can still be measured if sedimentation equilibrium is performed in media of different densities obtained by the addition of $H_2^{18}O$, D_2O, or $D_2^{18}O$ [21,27,175, 176], or by the use of detergent with densities similar to that of the medium [11,44]. In our former review [7] we have discussed not only the interest of knowing the state of aggregation of a solubilized membrane protein, but also the technical aspects of sedimentation equilibrium and sedimentation velocity within the particular context of a detergent and lipid binding protein. Here we want to point out some recent developments of these techniques. Firstly, a new Beckman analytical ultracentrifuge, the OPTIMA XLA, has become commercially available and is increasingly present in research institutes. Replacing the classical Model E, it is easier to set up and to use. Coupled with the centrifuge, a number of computer programs are now available for data analysis (e.g., see [177–182]). As examples of molecular mass determination of membrane proteins in detergent using the XLA we may cite [127,182,183]; furthermore monomer/dimer equilibrium constants in detergent have been determined (e.g., [44]). Secondly, preparative ultracentrifuges can be used to determine M_r by sedimentation equilibrium [184,185]; because the volume dependence of the time required to reach equilibrium necessitates the use of samples of less than 200 μl, table-top centrifuges such as Beckman airfuges and TL-100 are used. In Garrigos et al. [186] we demonstrate the use of the latter equipment to determine the M_r of bacteriorhodopsin solubilized in Triton X-100.

Finally, we may mention alternative techniques (although the equipments are not so readily available) to estimate molecular mass of solubilized membrane proteins: scanning transmission electron microscopy (STEM) which measures the number of electrons elastically scattered by the particle [157,187,193], small-angle X-ray or neutron scattering [32,47–49,188,189], and low-angle laser light-scattering photometry coupled with high-performance gel chromatography [154,190]. We note that the results of mass measurements by STEM are not very well correlated with the analytical ultracentrifugation data performed on the same complex [193], maybe because the conditions of experiments are very different: the former technique required removal of all non-volatile salts (water washing of the adsorbed particles) and freeze-drying of the sample. In fact, the use of detergent-free solution prior to freezing b_6f preparations in micelles yielded extremely polydisperse fields of particles in STEM [157]. Small-angle X-ray or neutron-scattering techniques can be used for detailed structural studies of the detergent–protein complex but require rather high protein concentrations (5–10 mg/ml) and put stringent requirements on the monodispersity of the samples which should be checked by analytical ultracentrifugation.

Acknowledgements

We wish to express our thanks to Dr A. Sanson for help with the calculation of the transmembrane perimeter of cytochrome oxidase, to Dr D. Picot for discussions about the neutrons diffraction and 3-D crystallization, to Dr B. de Foresta for suggestions to improve the manuscript, to Dorte Abildskov and Bitten Holm for clerical help and technical assistance, to Drs M. Roth and P. Timmins for the photographs shown in Fig. 6, and to the Association Française contre les Myopathies for financial support. The review was facilitated by the CNRS GDR 692 interdisciplinary network *Colloïdes en interaction* and by the biennial EMBO Practical Course 'Current Methods in Membrane Protein Research' at EMBL Heidelberg, Germany.

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