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Critical dependence of the solubilization of lipid vesicles by the detergent CHAPS on the lipid composition. Functional reconstitution of the nicotinic acetylcholine receptor into preformed vesicles above the critical micellization concentration

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Abstract

The critical concentration of free detergent $[D_w]^c$, which is necessary for lipid vesicle solubilization, is widely believed to be near to the critical micellization concentration (CMC) of the detergent. Here it is shown that $[D_w]^c$ and the critical detergent/lipid ratio $R^c(M)$ in the mixed micelles strongly depend on the lipid composition. In agreement with the concept of packing constraints, phospholipids with a large head-group were solubilized by the detergent CHAPS at low CHAPS concentrations, for example $[D_w]^c = 0.27$ mM for phosphatidyl–inositol and $[D_w]^c = 2.3$ mM for phosphatidylcholine, $T = 293$ K (20°C). In contrast, phospholipids (PL) with a small head-group required larger [CHAPS] values for (mixed) micelle formation: $[D_w]^c = 3.2$ mM for phosphatic acid (PA) and $[D_w]^c = 5.2$ mM for a mixture of palmitoyloleoylphosphatidylcholine (POPC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), POPC:PE:PG = 30:60:10 (mol.-%). Values obtained for the partition coefficient $K = R^c(M)/[D_w]^c$ ranged from $K = 0.12$ mM^{-1} for dimyristoylphosphatidylcholine to $K = 1.1$ mM^{-1} for (soy) phosphatidylcholine:phosphatidylglycerol (50:50). After addition of 30 mol.-% cholesterol (Ch) or with dipalmitoylphosphatidylcholine below $T = 308$ K (35°C), $[D_w]^c$ is > 10 mM, which is far above the CMC = 4 mM. This indicates that CHAPS micelles are formed before vesicle solubilization begins. The analysis of vesicle solubilization was a prerequisite for the controlled reconstitution of protein membrane proteins. AChR proteins reconstituted into preformed PL/Ch-vesicles at $[D_w] >$ CMC had a 4–5 time higher value of Li-influx compared to reconstitutions from completely solubilized lipid and proteins indicating a higher efficiency of right-side out AChR incorporation.

Keywords: Phospholipids; Cholesterol; Light scattering; Electron microscopy; Lateral pressure; Packing constraints

1. Introduction

The detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), which was specifically designed for membrane biochemistry [1], is often employed for protein solubilization from and reconstitution into the lipid phase. In addition, the development of drug carriers on the basis of lipids,

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detergents and other cosurfactants is a rapidly expanding field of research [2–4].

A model, which is commonly used to describe lipid vesicle–micelle transitions is characterized by (at least) three stages of solubilization: (i) binding of detergents to the lipid membranes, (ii) rupture of the detergent saturated vesicles resulting in the formation of mixed micelles in equilibrium with vesicles, or more general, with lamellar structures and (iii) a region were all lipids are solubilized in mixed micelles; see, e.g., Refs. [5–10]. However, the transformation to micelles is a matter of controversy. Structures intermediate between vesicles and micelles were proposed, like lamellar sheets, rod-like micelles or interdigitated structures, which depend on the nature of the surfactants used [7,11].

The transformation of vesicles to micelles by increasing detergent concentrations can be formally described as a (pseudo) phase transition [5,6]. Separating vesicular and micellar species by centrifugation [9,12] or gel permeation [13], it was shown that the effective molar ratio of detergent to lipid, $R = [D_b]/[L_T]$, at the transition, is higher in the mixed micelles ($R^c(M)$) than in the detergent saturated vesicles ($R^c(V)$). Here $[D_b]$ and $[L_T]$ denote the concentration of bound detergent and of total lipid, respectively. According to Lichtenberg [5] and Schurtenberger et al. [14], a partition coefficient K has been defined as

$$K = [D_b]/([L_T] \times [D_w]) = R/[D_w], \qquad (1)$$

which is related to R by the concentration of the free detergent in water $[D_w]$. At the phase transformation $[D_w] = [D_w]^c$ ideally remains constant, whereas K increases respective to the difference between $R^c(M)$ and $R^c(V)$.

When the critical total detergent concentration $[D_T]^c$ at a certain stage of the transformation is determined as a function of $[L_T]$, e.g. by turbidity measurements or light scattering, $R^c(M)$, $R^c(V)$ and $[D_w]^c$ can be evaluated according to Eq. (2) [5,6]:

$$[D_T]^c = [D_w]^c + R^c(M,V) \times [L_T]. \qquad (2)$$

In addition to structural changes of the microscopic aggregates, also macroscopic phase separations have been observed near the transformation of vesicles to micelles [6,7].

Reconstitution of bacteriorhodopsin at subsolubilizing detergent concentrations resulted in an asymmetric protein incorporation [15]. Asymmetric protein orientation is often advantageous for measurements of membrane transport or other functional assays. However, this reconstitution technique requires a detailed knowledge about the solubilization of vesicles by detergents. In addition, for investigations of protein–lipid interactions different lipid mixtures must be applied. So far, mainly phosphatidylcholin (PC) was used for the analysis of vesicle solubilization.

Here, the influence of the lipid head-group, of the hydrocarbon chains and of cholesterol on the solubilization of vesicles by CHAPS has been investigated. The results are compared with (i) the concept of packing constraints of the lipids and (ii) the lipid dependence of the lateral pressure.

2. Materials and methods

2.1. Vesicle preparation and solubilization

Soy bean phospholipids 99% pure (phosphatidic acid (PA), PC, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG)) and L$_\alpha$-palmitoyloleoylphosphatidylcholine (POPC) were purchased from Lipoid KG, Ludwigshafen, soy bean phospholipids (SBL, 20% lecithin) and phosphatidylinositol (PI) from Avanti Polar Lipids, L$_\alpha$-dimyristoylphosphatidylcholine (DMPC), L$_\alpha$-dipalmitoylphosphatidylcholine (DPPC), cholesterol (Ch) and CHAPS from Sigma. (a) Extrusion. The lipid was dissolved in chloroform and evaporated to a thin film in a round bottom flask. The dried lipid film (10 g/l) was dispersed in 10 mM Hepes (pH 7.4), 100 mM NaCl and 3 mM NaN_3, 20°C (buffer A) by rotation with a few glass beads and finally sonicated 1 min in a bath. After 5 freeze and thaw cycles the suspension was extruded through polycarbonate filters with 400 nm pores (11x). Alternatively, DPPC-vesicles were prepared at 45°C. For the solubilization of vesicles equal volumes of lipid and CHAPS solutions (at 2x the final concentration) were mixed, vortexed and incubated for 1 day. For pH 3–5 phosphate buffer was used instead of Hepes.

2.2. Determination of lipid composition

To separate the Ch crystals, the vesicles were solubilized with CHAPS and the remaining crystals were pelleted in a Biofuge A (Heraeus) 30 min at 13 000 rpm. The pellet was washed 2 times with buffer and solubilized in chloroform–methanol (1:1). Alternatively the crystals were separated by nucleopore filters (0.8 μm pores). Thin layer chromatography of surfactants was done as described earlier [16].

2.3. Light scattering

The solubilization of vesicles to micelles was visualized by light scattering at 90° with a Hitachi F-4010 fluorescence spectrophotometer, at 20°C, if not stated otherwise.

2.4. Flux measurements

Purification and reconstitution of *Torpedo californica* nicotinic acetylcholine receptor, as well as lithium flux measurements, were performed as described earlier [16]. The lipid concentration was 2 g/l, the protein concentration 0.1 g/l in all samples. For reconstitutions into detergent saturated vesicles the following CHAPS concentrations were used, depending on the lipid composition: POPC:PE:PG:Ch (27:54:9:10) 6.5 mM, (21:42:7:30) 8.1 mM, (15:30:5:50) 9.8 mM, POPC:PG:Ch (63:7:30) 1.6 mM. Carbamoyl choline induced influx measured with vesicles from (total) soy bean phospholipids and 10% cholesterol was taken as standard and set as 1 (100%).

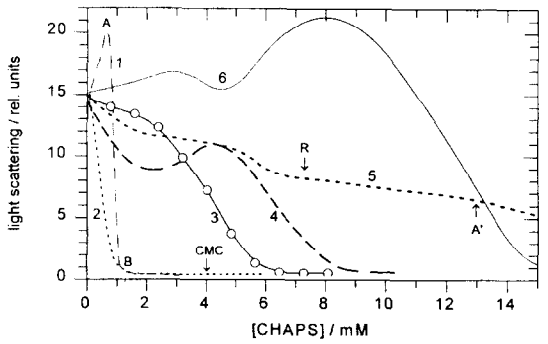

Fig. 1. Solubilization curves of lipid vesicles. Light scattering intensity (90°, $\lambda = 500$ nm) as a function of the CHAPS concentration. The lipid concentration is $[L_T] = 1$ g/l. The standard temperature for all lipids with unsaturated fatty acids is 293 K (20°C). Pure lipids and lipid compositions given as molar ratios: 1 = DMPC (283 K, 10°C); 2 = PG; 3 = PA; 4 = PE:POPC:PG (75:15:10); 5 = PE:POPC:PG:Ch (54:27:9:10); 6 = DPPC (291 K, 18°C). For the points A, A', B and R see the text.

2.5. Electron microscopy

Vesicles were negatively stained by uranyl acetate as described earlier [16]. Electron micrographs were viewed at 50 kV in a Zeiss TEM-109 electron microscope.

3. Results

3.1. Influence of the lipid composition on the solubilization curves

Increasing amounts of CHAPS were added to lipid vesicles until the light scattering intensity had dropped to a constant low value, near to that of the pure buffer solution (break point B, Fig. 1). The low scattering intensity indicated that all the lipid was solubilized in *small* mixed micelles (excluding lamellar sheets, cylindrical micelles etc.). The form of the solubilization curves, as shown in Fig. 1, changed substantially with the lipid composition. In the case of DMPC (curve 1) the three stages of the solubilization process are clearly separated by break point A at the peak of the curve (detergent saturated vesicles) and break point B at the baseline (mixed micelles).

The solubilization curves of PG (curve 2) and PI vesicles (similar curve not shown) decay without preceding peak or plateau and the solubilization is completed far below 4 mM CHAPS, the value of the CMC [16]. Therefore, the PI- and PG-vesicles react as if they are detergent saturated from the beginning. Here, but also in curve 3 (PA) break point A can hardly be assigned.

In contrast, vesicles enriched with PE or Ch were solubilized at much higher detergent concentrations. Additional stages in the solubilization process were indicated by a minimum or a point of inflection in the solubilization curve (curve 4 and 5). In the

Table 1

Characteristic parameters of vesicle solubilization. Critical concentration of free detergent $[D_W]^c$, molar detergent to lipid ratio R^c, partition coefficient K and AChR-mediated Li^+ influx determined for different lipid compositions. (M) and (V) denotes mixed micelles and detergent saturated vesicles, respectively. The Li^+ influx of AChR reconstituted with soy bean phospholipids and 10% cholesterol was used as reference and set as 1. All values are averages of 2 preparations and 2–3 samples each (S.D.: 3–12%)

No.	Lipid (mol.-%)	$[D_W]^c$ (M) (mM)	R^c (M)	K (M) (mM^{-1})	$[D_W]^c$ (M) (mM)	R^c (V)	K (V) (mM^{-1})	Relative flux values [a]	
								M	V
1	PC	2.3	1.0	0.43					
2	PI	0.27	0.17	0.6					
3	PG	0.45	0.44	1.0					
4	PA	3.2	1.8	0.56					
5	SM:PG (90:10)	2.1	0.83	0.40					
6	PC:PG (50:50)	0.74	0.84	1.1	0.5	0.33	0.66		
7	PE:PA (50:50)	4.2	1.2						
8	POPC:PG (90:10)	1.8	0.77	0.43					
9	POPC:PE:PG (60:30:10)	3.4	1.1	0.32					
10	POPC:PE:PG (30:60:10)	5.2	1.5		3.1	0.38	0.12		
11	POPC:PE:PG (15:75:10)	6.0	2.1						
12	Soy bean phospho lipids (SBL) [c]	3.2	1.5	0.47					
13	POPC:PE:PG:Chol (27:54:9:10)	8.3	3.0		6.5	1.0		0.23	1.3
14	POPC:PE:PG:Chol (21:42:7:30)	10.5	9.3		7.6	2.9		0.27	1.0
15	POPC:PE:PG:Chol (15:30:5:50)	10.6	17.0						leaky [b]
16	POPC:PG:Chol (81:9:10)	2.4	0.74	0.31			0.18		
17	POPC:PG:Chol (63:7:30)	4.7	1.2				0.36	0.27	
18	POPC:PG:Chol (45:5:50)	13.7	16.4						
19	PG:Chol (90:10)	1.1	0.60	0.55					
20	PG:Chol (70:30)	2.2	0.70	0.32					
21	PG:Chol (50:50)	10.6	9.9						leaky [b]
22	DMPC 10°C	1.0	0.12	0.12	0.6	< 0.05	< 0.1		
23	DMPC 27°C	1.0	0.37	0.37	0.6	0.1	0.16		
24	DPPC 18°C	12.5	4.5		6.1	1.1			
25	DPPC 27°C	9.5	3.2		5.0	0.62			
26	DPPC 35°C	4.4	2.1		2.2	0.37	0.17		
27	DPPC 40°C	2.1	0.8	0.38	0.9	0.1	0.11		

[a] The first flux value refers to reconstitution from mixed micelles (M), the second value to reconstitutions from preformed vesicles at subsolubilizing detergent concentrations (V).

[b] At 50% Ch part of the cholesterol precipitates as crystals upon detergent removal.

[c] From Ref. [16].

presence of Ch (curve 5) the transformation of vesicles to micelles is not as sharp as observed for phospholipids only. There is a characteristic step at 4–6 mM CHAPS, which is just above the CMC. The position of this decrease in light scattering was nearly independent of $[L_T]$ and obviously indicates the beginning of detergent micelle formation (note also the position of the minimum and maximum in curve 4 and 6 (DPPC), respectively). Breakpoint A' (the change of the gradient is difficult to see at this scale) moved considerably to higher values of $[D_T]$ with increasing $[L_T]$ and could be related to detergent saturated vesicles (see electron microscopy below). However, a detailed analysis of intermediate structure formation was not in the scope of this investigation.

For the same reason the determination of R^c and $[D_w]^c$ was limited to break point B for most of the lipid compositions. Here, at the end of the solubilization curve, the final value of the light scattering signal was generally obtained in minutes rather than hours, as in the transition region.

3.2. Parameters of vesicle solubilization, $[D_w]^c$, $R^c(M)$ and K

$[D_w]^c$ and $R^c(M)$ were determined by plotting $[D_T]^c$ as a function of $[L_T]$ (see Fig. 2 and Table 1). The values of $[D_w]^c$ and $R^c(M)$ are (inversely) correlated to the size of the lipid head-group. Lipids with a large head-group like PI and PG have low values

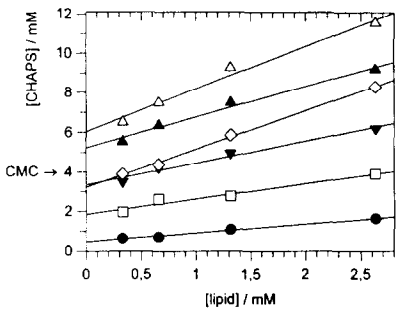

Fig. 2. Critical detergent concentration $[D_T]^c$ as a function of the lipid concentration $[L_T]$, 293 K (20°C). The lines reflect linear regression of the data according to Eq. (2) of the text. Lipid composition (molar ratio): ● PG; □ POPC:PG = 9:1; ▼ POPC:PE:PG = 6:3:1; ◇ PA; ▲ POPC:PE:PG = 3:6:1; △ POPC:PE:PG = 1.5:7.5:1.

of $[D_w]^c$ and $R^c(M)$, whereas PE and Ch stabilize the vesicles. At high lipid ratios of PE and/or Ch, $[D_w]^c$ exceeded considerably the value of the CMC of CHAPS. This was also found with DPPC below T_m. According to Eq. (2) the intercept on the ordinate yields the concentration of free detergent in water $[D_w]^c$. However, when the intercept is $[D_w]^c >$ CMC, it cannot represent the concentration of detergent monomers. Rather, the participation of detergent micelles in the solubilization of vesicles has to be considered (see below). PE and Ch have a synergistic effect on the vesicle stability since the increase of $[D_w]^c$ and $R^c(M)$ after addition of 10% Ch is larger for lipid composition No. 10 compared to composition No. 8 of the Table.

To investigate charge effects, PA-vesicles were solubilized also at pH 5, 4 and 3. At pH 5 and pH 4 no difference was observed in the solubilization compared to pH 7.4. At pH 3 the solubilization was a two-step process. In contrast to higher pH values, a first decrease of the light scattering signal was measured already at low CHAPS concentrations. The value of $[D_w]^c$ increased with 12%.

To investigate the influence of acyl chain length and the physical state of the membrane the synthetic lipids DPPC and DMPC were used. The value of $R^c(M) = 0.12$ mM for DMPC is still a little smaller than that obtained for PI. Above T_m, $R^c(M)$ is tripled with no change in $[D_w]^c = 1.0$ mM. In contrast, DPPC shows a large change in micelle formation at T_m (which is below 40°C for detergent saturated vesicles [17]). Above T_m, the parameters of solubilization equal those of PC at 20°C. Below T_m, a dramatic increase of $[D_w]^c$ and $R^c(M)$ is observed up to $[D_w]^c = 12.5$ mM and $R^c(M) = 4.5$. Even $[D_w]^c(V) = 6.1$ mM (detergent saturated vesicles) is clearly above the CMC.

The partition coefficient K was obtained from $[D_w]^c$ and $R^c(M)$ for each lipid composition as defined in Eq. (2), provided the value of the unbound CHAPS was $[D_w]^c <$ CMC. According to the observed correlation of $[D_w]^c$ and $R^c(M)$ the differences between the K values were relatively small. Most of the lipid compositions had a value of $K =$ 0.3–0.6 mM^{-1} with the exception of DMPC ($K =$ 0.12 mM^{-1}), PG ($K = 1$ mM^{-1}) and PC:PG (50:50, $K = 1.1$ mM^{-1}). It is clearly seen from Table 1, that K decreases with increasing concentration of PE or

Ch, which is known to have a condensing effect on the bilayer. Similarly K becomes smaller below T_m, when DMPC is in the liquid condensed state. As expected, $R^c(V)$ and the partition coefficient $K(V)$ determined at breakpoint A are lower than the corresponding values $R^c(M)$ and $K(M)$ for the mixed micelles.

3.3. Reconstitution of acetylcholine receptor

Since several lipid mixtures were solubilized only above the CMC, it was interesting to know, if membrane proteins could insert into these 'rigid' membranes at subsolubilizing detergent concentrations (marked R in Fig. 1). Isolated detergent solubilized acetylcholine receptor (AChR) proteins from *Torpedo californica* were incubated with preformed lipid vesicles as described in the Methods. The functional reconstitution of the AChR was analyzed by carbamoyl choline induced lithium influx. Since the AChR needs Ch for its proper function, only lipid compositions with Ch were applied. For comparison, the receptors were also reconstituted from completely solubilized micelles. In the latter case, the lithium influx for all artificial lipid compositions was only about 1/3 of the value measured in the presence of soy bean phospholipids (SBL, reference value). In contrast, at subsolubilizing detergent concentrations, the PE containing lipid compositions yielded a higher flux value (1–1.3 ×, data set V). The higher uptake of Li^+ can be partly attributed to the larger size of the vesicles formed by extrusion, as judged from the non-induced influx (without carbamoylcholine, data not shown). When Ch reached 50% of the lipid concentration the carbamoyl choline induced influx was only 5–10% higher than that without agonist, independent of the reconstitution method. The formation of cholesterol crystals during reconstitution indicated that the lipid was supersaturated with Ch. It is supposed that vesicle membranes with this lipid composition have an increased ion permeability. Ch was determined as the only compound of the isolated crystals by thin layer chromatography.

3.4. Structural analysis by microscopy

As PI was solubilized at a very low detergent concentration, it was interesting to know if PI itself formed vesicles or rather larger micellar structures. According to the electron micrograph (Fig. 3a) actually vesicles were formed. Although the collapsed appearance seems to be a characteristic property of the PI-vesicles, an influence of the staining procedure cannot be excluded. In addition, PG-vesicles were found to retain NaCl (data not shown).

To proof the existence of vesicles above the CMC, electron microscopy was used. The Ch–phos-

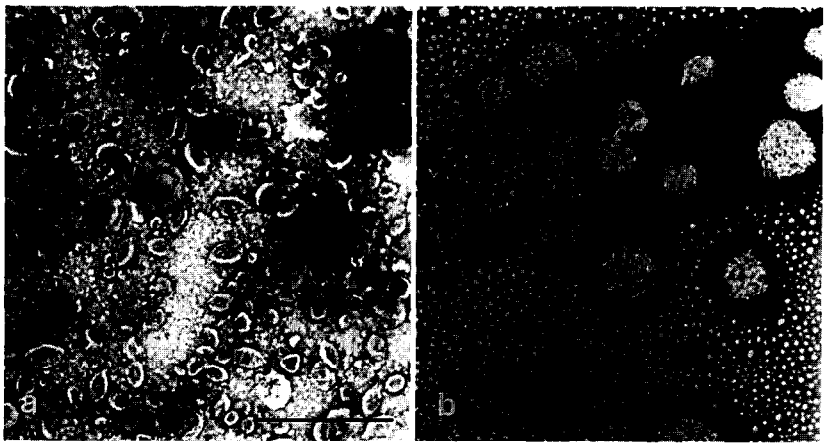

Fig. 3. Negative stain electron micrographs of lipid vesicles. (a) PI-vesicles and (b) vesicles of a lipid mixture with detergent, 1 g/l PE:PC:PG:Ch (42:21:7:30) + 5 g/l (≈ 8 mM) CHAPS in the buffer: 10 mM Hepes, pH 7.4, 150 mM NaCl, 293 K (20°C). Some of the PI vesicles have a collapsed appearance and are possibly shrunk during the staining procedure. The bar represents 1 μm.

pholipid compositions No. 13, No. 14 and No. 17 (Table 1), were used for reconstitution at subsolubilizing detergent concentrations. The stage in the solubilization curve, which corresponds to the lipid/detergent composition in AChR reconstitution is marked by R (Fig. 1, curve 5). The electron micrograph (Fig. 3b) clearly shows that vesicles still exist at this stage, where $[D_T] = 8$ mM > CMC (lipid composition No. 14). In addition, all vesicle suspensions, which were used for AChR reconstitution at $R < R^e(V)$, were analyzed in a darkfield microscope. In the darkfield microscope vesicles of diameter 200–400 nm can be well visualized and differentiated from micellar structures. The addition of subsolubilizing concentrations of CHAPS did not noticeably change the appearance and the amount of the vesicles.

4. Discussion

In this investigation light scattering was used to study the transformation of lipid vesicles to micelles in the presence of the detergent CHAPS. Complementary methods as centrifugation, ^{31}P NMR or fluorescence resonance energy transfer have corroborated the assignment of the breakpoints A, B to detergent saturated vesicles or lamellar sheets ($R^e(V)$) and to mixed micelles ($R^e(M)$), respectively [7–9]. Regarding breakpoint B, it is obvious that these results, which were obtained for lecithin and different detergents, can be applied to other lipid compositions, too.

The values of $R^e(M)$ and $[D_w]^e$ determined at break point B can be taken as a quantitative measure for the 'solubility' of a lipid composition in an aqueous CHAPS solution. Furthermore, the data can be directly used to calculate the minimal detergent concentration, which is necessary for complete lipid solubilization and subsequent reconstitution at any (moderate) lipid concentration. Although $[D_T]^e$ increases linearly with $[L_T]$ also above the CMC, it is clear that there is no simple equilibrium between bound and free detergent as assumed in Eqs. 1 and 2. Since this issue is not primarily essential for protein reconstitution a detailed analysis of pseudophases was considered for a separate investigation. For protein reconstitution in detergent saturated vesicles also the begin of micelle formation (breakpoint A) should be known. However, especially those lipid compositions, which were solubilized at high [CHAPS], show additional stages of solubilization, as indicated by light scattering. For lipid compositions containing Ch an additional break is seen in the solubilization curve at 4–6 mM CHAPS (curve 5). The position of this break was relatively independent of $[L_T]$. Therefore, it probably reflects the formation of detergent micelles and not the disintegration of vesicles. Further evidence was taken from darkfield microscopy, which showed that vesicles were stable up to break A'. In addition the existence of vesicles at 8 mM CHAPS was proved by electron microscopy (Fig. 3).

Although intermediate structure formation was not in the focus of this work, the following observations, which are important for reconstitution, will be shortly discussed. A peak in the solubilization curve, as shown for DPPC (curve 1), was observed with all pure phosphatidylcholine vesicle preparations. The increase of turbidity, which has been attributed to vesicle or lamellar sheet expansion, aggregation or transformation to cylindrical micelles [8,12,18,19], only occurred in preparations without negatively charged lipids. When charged detergents were used for solubilization, an increase in particle size was not found either [20]. Since aggregation of particles could have concealed other structural changes, at least 10% negatively charged phospholipids were applied in most of the experiments. Interestingly, also the separation of the system octyl glucoside/lecithin into two macroscopic phases can be prevented by addition of $\geqslant 10\%$ negatively charged lipids [6]. Therefore, the application of negatively charged lipids seems to be generally advantageous for protein reconstitution.

Protonation of PA should be nearly complete at pH 3 since in membranes $pK_1(PA) = 4$ [21]. Protonation decreases the hydration of the head-group, and hence the effective surface area [22]. Thus, at pH 3 participation of H_{II} structures may be involved, which are not useful for vesicle reconstitution. Calorimetry measurements of DPPC in the presence of cholate and deoxycholate by Bayerl et al. [17] yielded a partition coefficient $K = 0.048$ mM^{-1} and 0.34 mM^{-1}, respectively. The former value is only two times lower than the value of $K(V)$, which was obtained for DPPC and CHAPS here at 40°C. The

much larger K value of deoxycholate can be attributed to the lower solubility of its steroid backbone in water [17].

4.1. Categories of solubilization

According to the values of $R^e(M)$ and $[D_w]^e$ the solubilization of vesicles by CHAPS can be divided into two categories:

Lipids, which are solubilized below the CMC of CHAPS, i.e. $[D_w]^e < CMC$. In this case $R^e(M) \leqslant 1.5$ can adopt only moderately high values. The detergent can penetrate into the bilayer and bind to an intercalating binding site [17]. The transformation to mixed micelles probably involves lamellar sheets as intermediate structures [7,10]. In spite of the structural differences between lipids and detergents, apparently ideal mixing behavior was found in the case of lecithin and octyl glucoside [23]. The head-group dependence of the $R^e(M)$-values is in line with the concept of packing constraints [24–26]; the results suggest that the average surfactant parameter $p = v/\sigma l$ of the lipids and CHAPS is similar in the different mixed micelles at the (pseudo) phase transformation. Here v is the volume of the hydrocarbon moiety, l the chain length and σ is the effective head-group area. This type of solubilization is in agreement with the model presented in the introduction.

Lipids, which are solubilized above the CMC. The critical value of CHAPS not bound to lipid is $[D_w]^e > CMC$. The detergents bind to the interface of the bilayer, but are not (or not enough for vesicle rupture) intercalating between the lipids. Above a critical detergent concentration (which might be slightly below the CMC of pure detergent solutions [17]) detergent micelles are formed, which probably can bind reversibly to the vesicles and take up low amounts of lipid. The low solubility of lipid in the detergent micelles is reflected by the high values of $R^e(M)$. A similar model of vesicle solubilization was proposed for the first step of Ch-crystal formation in bile acids from lipid vesicles supersaturated with Ch [27]. It was found that the phospholipids were selectively extracted from the vesicles into the detergent micelles, resulting in an accumulation of Ch in the vesicles [27]. Here, the formation of Ch-crystals was also observed in the presence of CHAPS at a Ch/phospholipid ratio $\geqslant 50\%$. A similar concept was also proposed by Bayerl et al. [17] for the solubilization of DPPC by deoxycholate at $[L_T] > 10$ mM. However, at $[L_T] = 1.5$ mM, DPPC vesicles were solubilized below the CMC. In contrast, CHAPS could not solubilize DPPC vesicles below the CMC at $T \leqslant 27°C$ ($[L_T] = 0.34$ mM). At $\geqslant 35°C$ the solubilization of DPPC can be assigned to the first category of micelle formation; here, no indication was found for a different solubilization mechanism of CHAPS at low and high values of $[L_T]$.

4.2. The concept of lateral pressure

Comparing the partition coefficients presented in Table 1, a decrease of K is observed with increasing concentrations of Ch and PE. These lipids are known to condense the bilayer and increase the lateral pressure [28]. Naumann et al. [29] have shown that the partition of detergents between a DPPC monolayer and the subphase critically depends on the lateral pressure π. Above $\pi \approx 25$ mN/m all detergents are squeezed out of the monolayer. Measurements with DMPC showed that the depletion of the detergent into the subphase is not necessarily connected with the formation of the liquid condensed phase [29]. This finding well compares with the above data of the solubilization of DMPC and DPPC. DMPC was solubilized at the same value of $[D_w]^e = 1$ mM below and above T_m, whereas DPPC could not be solubilized at $[D_w]^e < CMC$ below T_m. For DPPC a higher lateral pressure is expected due to the increase in chain length. (Nevertheless, packing constraints may as well contribute to the different solubilization of DPPC and DMPC).

Assuming a molecular area of CHAPS $\sigma \approx 0.5$ nm^2 in a lipid layer, one can calculate the additional (molar) work of surface expansion ΔG_π^o, which is needed for the insertion of CHAPS after an increase of the lateral pressure $\Delta\pi$ as:

$$\Delta G_\pi^o = \Delta\pi \cdot \sigma \cdot N_A \qquad (3)$$

where N_A denotes the Avogadro's constant. For $\Delta\pi = 1$ mN/m $\Delta G_\pi^o \approx 0.3$ kJ/mol. The increase of π for phospholipid vesicles after addition of Ch can well be $\Delta\pi > 5$ mN/m, as judged from the lateral pressure of a monolayer in equilibrium with vesicles in the subphase (H. Schindler and Th. Schürholz,

unpublished results). Because $K = \exp(-\Delta G^{\circ}/RT)$ is connected with the standard value of the molar Gibbs free energy change of reaction $\Delta G^{\circ} = \Delta G_0^{\circ} + \Delta G_{\pi}^{\circ}$ we obtain $K = K_0 \exp(-\Delta G_{\pi}^{\circ}/RT)$. For $\Delta\pi = +5$ mN/m this yields an about two-fold decrease in K, which is in the order of the observed changes at $[D_w]^c < CMC$. It should be noted, however, that the values of K determined at $R^c(V)$ include detergent binding to the vesicle interface, which is dominated by different forces.

4.3. Protein reconstitution

Similar to detergent incorporation, the insertion of proteins into membranes and monolayers critically depends on the lateral pressure as shown by phospholipase activity or AChR uptake [30,31]. Therefore, the incorporation of AChR into the vesicles at high concentrations of PE and Ch could have been hindered by the high lateral pressure. On the other hand, Ch was found to facilitate protein incorporation by specific interaction [1008] and PE is known to facilitate fusion. In line with the solubilization of 'rigid' vesicles above the CMC, it was found that the dissociation of protein aggregates can require > 10 mM CHAPS [16]. Therefore, a high detergent concentration during reconstitution may be a prerequisite for a homogeneous protein distribution. In addition, incorporation of proteins into preformed vesicles at $[D_w] < [D_w]^c(V)$ avoids the formation of micellar structures with different lipid/surfactant compositions [13,19]. The divergence of micellar structures and compositions is expected to contribute to the inhomogeneous protein/lipid ratios found in reconstituted vesicles [16]. However, some specific extraction of lipid from the vesicles is expected also below $R^c(V)$ [27]. Anyhow, the data have shown, that the functional reconstitution of AChR in lipid vesicles can be favorably done at subsolubilizing [CHAPS] in the presence of Ch and PE. In addition to an improved protein distribution and the relatively large size of the preformed vesicles, the expected increase in right side out orientation probably contributed to the high flux values.

The parameters of vesicles solubilization by CHAPS determined here for different lipids and lipid mixtures may serve as a basis for further reconstitution strategies.

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