Selective detergent-extraction from mixed detergent/lipid/protein micelles, using cyclodextrin inclusion compounds: a novel generic approach for the preparation of proteoliposomes

Willem J. DEGRIP[1], Jenny VANOOSTRUM and Petra H. M. BOVEE-GEURTS

Department of Biochemistry – FMW 160, Institute of Cellular Signalling, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

A novel generic approach is described for the selective extraction of detergents from mixed detergent/lipid/protein micelles for the preparation of proteoliposomes of defined lipid–protein ratio. The approach is based on the much higher affinity of inclusion compounds of the cyclodextrin type for detergents in comparison with bilayer-forming lipids. This approach has distinct advantages over other procedures currently in use. It produces good results with all detergents tested, independent of type and critical micelle concentration, and appears to be generally applicable. It yields nearly quantitative recovery of membrane protein in the proteoliposome fraction. Finally, no large excess of lipid is required; a molar ratio of lipid to protein of 100 to 1 already produces proteoliposomes with functional membrane protein, but higher ratios are well tolerated. The size of the vesicles thus obtained depends on the detergent used. Separation of the resulting proteoliposomes from the detergent–cyclodextrin complexes was most easily achieved by centrifugation through a discontinuous sucrose gradient. A variety of detergents was tested in this procedure on the bovine rod visual pigment rhodopsin in combination with retina lipids. In all cases good yields of proteoliposomes were obtained, which contained fully functional rhodopsin.

INTRODUCTION

Interest in membrane proteins has surged over the last two decades. This is the result of growing awareness that membrane-bound processes constitute very important control and regulation sites in cellular physiology and communication, as well as of the rapid progress in recombinant DNA-technology. Membrane proteins, hitherto not accessible for detailed structural and biochemical analysis because of their low expression levels in native tissue, can now be accessed through functional over-expression. Nevertheless, whatever their source, a major bottle-neck in studying membrane proteins remains their purification. Almost inevitably, detergents have to be used to resolve the membrane suprastructure into small units, mixed micelles consisting of detergents, membrane lipids and individual membrane proteins, which are amenable to selective purification [1,2]. At the same time, this presents a major drawback. Transfer of membrane protein from its membrane environment to a mixed micellar environment nearly always results in a loss of structural stability and of functional properties [1,2]. The extent of this loss is strongly detergent-dependent, but even very 'mild' detergents like digitonin or dodecyl maltoside cannot fully mimic the membrane environment [3–5]. These effects are usually aggravated upon purification due to loss of lipid. In addition, lipid preference or vectorial/interactive properties of a membrane protein (transport, oligomerization) cannot be studied in a mixed micelle. Hence, for most structural and functional studies it is essential that, subsequent to purification, the membrane protein is transferred back into a membrane environment (reconstitution).

For reconstitution of membrane proteins a variety of techniques have been proposed, but only a limited number have gained widespread use. A first choice usually is removal of detergent from mixed micelles by dialysis [1,2,6,7], gel filtration or dilution [2,8]. This approach is only feasible, however, for detergents with a high CMC ($\geqslant$ 5 mM as a rule of thumb), and in case of gel filtration may suffer from low recoveries. Unfortunately, this approach is not appropriate for quite popular, relatively mild detergents like digitonin, Triton X-100 and dodecyl maltoside, which have a CMC below 0.5 mM [9–11]. Another option is to extract the detergent by adsorption to hydrophobic beads [1]. This is particularly suitable for detergents with a low CMC, but suffers, unless very special precautions are taken [7], from appreciable losses of membrane protein, which can only be mitigated by using a large excess of lipid. In fact, there is no simple, generally applicable approach for reconstitution of membrane proteins, and detergents with low CMC ($<$ 1 mM) are particularly problematic in this respect.

Lately, compounds of the cyclodextrin class (cyclic oligoglucosides) have received much attention because of their ability to solubilize hydrophobic molecules as inclusion complexes [12–14]. Many-fold applications are developed including chiral separation, drug carrier, drug formulation, steroid and fatty acid extraction and protein folding [15–23]. While we were testing selective extraction of retinals from membranes by means of cyclodextrins, we observed that these compounds only have low affinity for diacylphospholipids (J. VanOostrum, P. H. M. Bovee-Geurts and W. J. DeGrip, unpublished work). Hence, we decided to test whether the affinity of cyclodextrins for detergents was sufficiently high to allow selective extraction of detergents from mixed micelles and produce proteoliposomes without significant loss of lipid and membrane protein. The relevant results are presented here. We demonstrate that, with a proper combination of detergent and cyclodextrin, cyclodextrin-mediated extraction of detergent does provide a novel and

Abbreviations used: CMC, critical micelle concentration; DoM, dodecyl maltoside; DTE, dithioerythritol; FT-IR, Fourier transform infrared; HOM-β-cyclodextrin, heptakis-2,6-di-*O*-methyl-β-cyclodextrin; ROS, rod outer segments; TLC, thin layer chromatography; PAGE, polyacrylamide gel electrophoresis; ANS, 8-anilino-1-naphthalene-sulphonate; T, transducin.

[1] To whom correspondence should be addressed.

generic approach for the production of functional proteoliposomes, independent of type and CMC of detergent.

EXPERIMENTAL

Materials

ANS (8-anilino-1-naphthalene-sulphonate) was from Eastman-Kodak. α-Cyclodextrin (cyclohexaamylose), β-cyclodextrin (cycloheptaamylose) and heptakis-2,6-di-*O*-methyl-β-cyclodextrin (HOM-β-cyclodextrin) were obtained from Aldrich. CHAPS(O), Triton X-100, Ammonyx LO (*N*,*N*-dimethyldodecylamine-*N*-oxide), cholate, HECAMEG [methyl-6-0-(*N*-heptylcarbamoyl-α-1)-glycopyranoside] and γ-cyclodextrin (cyclooctaamylose) were obtained from Fluka. *N*-dodecyl-β1-maltoside (DoM) and *n*-nonyl-β-1-glucoside (nonylglucose) were prepared and purified as described before [11] or obtained from Anatrace. Zwittergent-314 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulphonate) was from Calbiochem and $C_{12}E_{10}$ (polyoxyethylene-10-laurylether) from Sigma. These and all other chemicals were of the highest purity available.

Throughout this manuscript a single buffer was used (buffer A: 20 mM Pipes, 130 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM EDTA, 1 mM DTE, pH 6.5). However, any other buffer system between pH 5.0 and 7.5 can be used. pH-values outside this range have not been tested, but should theoretically not present a problem as long as the protein is stable under those conditions.

Isolation of rod outer segments and functional assay of rhodopsin

Bovine eyes were obtained from a local slaughterhouse and kept in a dark container until dissection. Rod outer segment (ROS) membranes were isolated under dim red light (Schott RG610 cut-off filter) basically as described [24]. In short, retinas were dissected from the eyes and subjected to mild homogenization in buffer A containing 23% (w/w) sucrose (0.5 ml/retina). The homogenate was filtered through Nylon maze (100 mesh) and the filtrate layered on top of a 23–36% (w/w) continuous sucrose density gradient (7 ml filtrate per 30 ml gradient volume). After centrifugation (2 h, 10 °C, 100000 ***g***) the ROS band was collected, diluted with one volume of buffer A and the ROS were pelleted (10 min, 4 °C, 2500 ***g***) and washed twice with deionized water (20 min, 4 °C, 160000 ***g***). The resulting photoreceptor membranes were stored in light-tight containers at −80 °C under Argon. Their A_{280}/A_{500} ratio was in the range 2.0–2.2 and their molar ratio of phospholipid to rhodopsin was 70 ± 5.

Photochemical functionality of rhodopsin preparations was assessed from the late steps in its photocascade (Meta I ⇄ Meta II equilibrium and Meta II → Meta III transition) by means of UV/Vis spectroscopy as described before [25]. In short, the rhodopsin sample was diluted into buffer A to a rhodopsin concentration of 1–2 μM. A 'dark' spectrum was recorded (700–250 nm, Perkin–Elmer lambda 15 recording spectrophotometer) and the cuvette was then illuminated (10 s, 75 W tungsten bulb, Schott OG530 and KG1 cut-off filters) bleaching about 60% of the rhodopsin. The first 'light' spectrum was then recorded and the subsequent thermal reactions were monitored by recording spectra every 3 min until Meta II decay had levelled off. Under these conditions, native rhodopsin produces a Meta I/Meta II equilibrium with 15 ± 5% Meta I and shows a half time of 6.5 min for Meta II decay. These functional characteristics are quite dependent on a lipid micro-environment and are very strongly perturbed by solubilization into micelles ([5,26–28], P. H. M. Bovee-Geurts and W. J. DeGrip, unpublished): the Meta I → Meta II transition then proceeds fully to Meta II, and Meta II decays rapidly and largely into opsin + free retinal.

Occasionally, also G-protein (transducin) activation was tested for rhodopsin preparations, although this property is less sensitive to the micro-environment. For this purpose three different assays were used: a spectroscopic Meta I–Meta II shift assay, which measures the shift in the Meta I ⇄ Meta II equilibrium due to binding of transducin (T) to Meta II [29,30], a GTP-T_α release assay measuring increase in fluorescence [31,32], and a potentiometric proton release assay [33], which measures cyclic GMP hydrolysis by the rod phosphodiesterase, the target enzyme of transducin (Rh* → T* → PDE*), modified as described [34].

CMC-measurement and CMC-shift assay

The CMC of all detergents was determined through the increase in fluorescence quantum yield of ANS due to incorporation in micelles upon micelle formation [11]. Briefly, buffer B (10^{-5} M ANS in 20 mM Pipes, pH 6.5) was set at 0% fluorescence yield [Shimadzu RF-520 spectrofluorophotometer; excitation: 360 nm (3 nm band-width), emission: 450 nm (5 nm bandwidth)]. Buffer B containing detergent (50–100 mM) was set at 100% fluorescence yield (buffer C). Then buffer B was titrated with buffer C yielding a graph of relative fluorescence versus detergent concentration (Figure 1). The CMC is determined by extrapolation of the linear part of this graph.

The CMC-shift assay measures the apparent shift of the CMC in the presence of a fixed cyclodextrin concentration due to complex formation of detergent and cyclodextrin preventing detergent association into micelles. This assay is performed exactly as described above, except that buffer B and C both contain a specified concentration of cyclodextrin (1, 5, 10 or 20 mM). We hereby define the relative CMC-shift as the ratio of the measured CMC-shift and the corresponding cyclodextrin concentration. Hence this relative CMC-shift can vary from 0 to 1.

Preparation of proteoliposomes based upon detergent complexation by cyclodextrins

Membrane preparations were dissolved in buffer A, containing 5 mM DTE and 20 mM detergent, up to a protein concentration of 1.5 mg/ml. Although protein concentrations between 0.1 and 1.5 mg/ml gave satisfactory results, higher recoveries were obtained for concentrations > 0.4 mg/ml. Lipids were isolated from bovine retina as described before [35]. The required aliquot was dried in a stream of nitrogen, dissolved in methanol ($\geqslant 20$ mg/ml) and added to 20 volumes of buffer A + detergent. The lipid solution was subsequently mixed with the membrane protein solution in such a ratio that the required molar lipid to protein ratio was acquired. This ratio was varied between 50 and 300. All solutions were kept on ice and under argon. Proteoliposomes were then generated by stepwise addition of solid cyclodextrin (5–10 mM steps). Each step was followed by a 15–30 min incubation on ice. Proteoliposome formation is indicated by an increase in turbidity, which is easily observed, and starts when the molar ratio of cyclodextrin to detergent approaches 1 (range 0.8–1.2). A final incubation on ice then completes vesicle formation.

If the detergent concentration is known exactly, cyclodextrin may be added in one step in a 1/1 molar ratio to detergent, followed by a 30 min incubation on ice. Preliminary data indicate that the one-step approach yields somewhat smaller vesicles, but vesicle-size largely depends on the detergent used (see Results).

For separation of proteoliposomes from detergent–cyclodextrin complexes centrifugation through a sucrose step-gradient was employed. The latter consisted (bottom to top) of a 45 % (w/w) sucrose layer, a 20 % (w/w) sucrose layer, a 10 % (w/w) sucrose layer and a 10 % (w/w) sucrose layer containing 10 mM cyclodextrin, in a volume ratio 2/3/2/2. The proteoliposome suspension (same volume as top layer) was carefully layered on top of the gradient. After centrifugation (12–16 h, 4 °C, 200000 ***g***) the proteoliposomes have migrated into the 20 % or on top of the 45 % sucrose layer. The detergent–cyclodextrin complexes remain on top of the gradient.

The proteoliposome band is collected, diluted with several volumes of buffer A and pelleted (2 h, 4 °C, 200000 ***g***). The pellets are routinely stored at −80 °C.

Structural analysis

The distribution of cyclodextrin–detergent complexes over the gradient was analysed by TLC, using the procedure of Colarow for separation of lipids [36]. This simultaneously yields the phospholipid distribution. For qualitative detection plates were sprayed with 0.1 % ANS in water and examined under near-UV light (350 nm).

Residual cyclodextrin and detergent in the final proteoliposome preparation were analysed by FT-IR spectroscopy ([6]; A. Pistorius, P. Bovee-Geurts and W. DeGrip, unpublished). Calibration curves for the detergents and cyclodextrins were prepared in ROS membranes as described [6] and allow direct estimation of the ratio of these compounds to phospholipids present in any vesicle preparation. The proteoliposomes collected from the sucrose step-gradient were pelleted, washed twice with distilled water to remove sucrose and deposited as a membrane film on AgCl-windows [6] for FT-IR analysis.

Orientation of rhodopsin in the proteoliposomes was determined by limited proteolysis with proteinase K followed by SDS/PAGE analysis [37]. Since (proteo)-liposomes from retina lipids are relatively permeable [35], penetration of proteinase K into the vesicles was prevented by using immobilized enzyme (Merck A.G.).

The ultrastructure of proteoliposomes, generated as described above, was examined by transmission electron microscopy. Hereto, aliquots of sucrose fractions were treated with glutaraldehyde [2 % (w/v) final concentration] for at least 1 h at 4 °C. The fixed material was then pelleted (30 min, 4 °C, 150000 ***g***). The pellets were rinsed with phosphate-buffered saline and fragments of the pellets were stained with 1 % (w/v) osmium tetraoxide, dehydrated with ethanol and embedded in Epon LX112. Thin sections were analysed in a Philips EM410 electron microscope.

RESULTS

Interaction of cyclodextrins with detergents strongly depends on their hydrophobic moiety

If a cyclodextrin should be able to extract detergent molecules from mixed micelles through formation of an inclusion complex, it also should be able to prohibit association of detergent monomers into micelles. We devised a CMC-shift assay to address this aspect (Figure 1). Here, detergent is titrated into a solution containing a fluorescent probe (ANS) and a fixed concentration of cyclodextrin. The probe reports on micelle formation. If the association constant of the detergent–cyclodextrin complex is high enough, most detergent-monomers will be present in such a complexed state, and will not be able to associate into micelles, until the detergent concentration approaches the cyclodextrin concentration. Assuming formation of a 1:1 complex [38], the CMC of the detergent will apparently shift with a value close to the cyclodextrin concentration and the observed shift will obey a linear relationship with the cyclodextrin concentration.

These assumptions were borne out by the following experiments. A variety of detergents, differing in hydrophobic moiety and in size and charge of the headgroup (Table 1), were tested in their interaction with α-cyclodextrin, HOM-β-cyclodextrin and γ-cyclodextrin. Pilot experiments demonstrated that β-cyclodextrin behaved identically to HOM-β-cyclodextrin, but the latter has a much higher solubility in aqueous solution [14] and

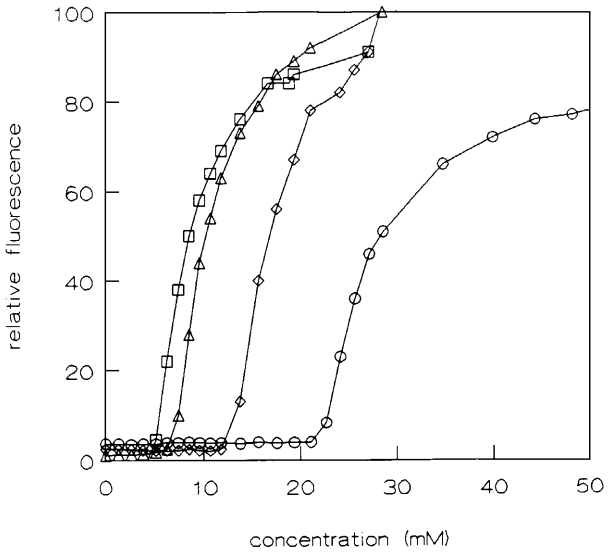

Figure 1 CMC-shift assay to probe detergent–cyclodextrin interaction

A typical example is given for the combination nonylglucose + α-cyclodextrin. Micelle formation is probed by increase in ANS-fluorescence. Buffer B (no detergent) is set at 0 % fluorescence and titrated with buffer C (100 mM detergent; set at 100 % fluorescence). Detergent concentration is on the *x*-axis. Extrapolation of the linear part of the resulting curve gives the value for the CMC. The assay was done in the presence of the following α-cyclodextrin concentrations (present in buffer B and C): 0 mM (□), 1 mM (△), 10 mM (◇) and 20 mM (○).

Table 1 Characteristics of detergents used in this study

Detergent	Hydrophobic moiety	Headgroup	CMC* (mM)
HECAMEG	Alkyl-chain (C7)	Nonionic (glucoside)	22
Nonylglucose	Alkyl-chain (C9)	Nonionic (glucoside)	6.5
Ammonyx LO	Alkyl-chain (C10)	Zwitterionic (*N*-oxide)	0.07
Dodecylmaltose	Alkyl-chain (C12)	Nonionic (maltoside)	0.16
$C_{12}E_{10}$	Alkyl-chain (C12)	Nonionic (polyoxyothylene)	0.02
Zwittergent-314	Alkyl-chain (C14)	Zwitterionic (ammonio-propane-sulphonate)	0.04
Triton X-100	Alkyl-chain + phenyl-group	Nonionic (polyoxyethylene)	0.13
CHAPS(O)	Sterol-derivative	Zwitterionic (ammonio-propane-sulphonate)	6.0
Cholate	Sterol-derivative	Negatively charged (carboxylate)	5.5†

* CMC-data from [9–11] or determined as described [11].
† In buffer A. The CMC of cholate is strongly pH- and ionic-strength dependent.

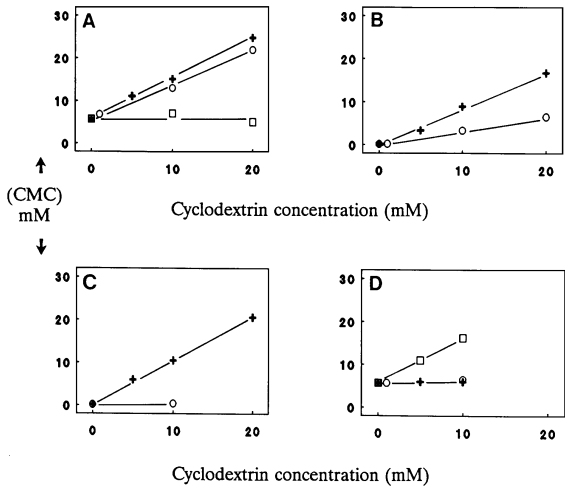

Figure 2 CMC-shift for four detergents showing the four patterns observed

The CMC measured in the presence of a fixed cyclodextrin concentration is plotted against that cyclodextrin concentration. The slope of the corresponding linear curve is the relative CMC-shift. (**A**), β-1-nonylglucose; (**B**), β-1-dodecylmaltose; (**C**), Triton X-100; (**D**), CHAPS(O). ○, α-cyclodextrin; +, HOM-β-cyclodextrin; □, γ-cyclodextrin.

Table 2 Affinity of the detergents from Table 1 for cyclodextrins*

Detergent	Cyclodextrin type		
	α	β	γ
HECAMEG	±	+	−
Nonylglucose	±	+	−
Ammonyx LO	±	+	−
Dodecylmaltose	±	+	−
$C_{12}E_{10}$	±	+	†
Zwittergent-314	±	+	−
Triton X-100	−	+	†
CHAPS(O)	−	−	+
Cholate	−	−	+

* +, high affinity ($K_a \geqslant 10^4$ M^{-1}); ±, intermediate affinity (K_a 1–5 × 10^3 M^{-1}); −, low affinity ($K_a < 10^3$ M^{-1}).

† The combination of Triton X-100 or $C_{12}E_{10}$ with γ-cyclodextrin resulted in a turbid sample.

has been used for all experiments described here. Four patterns were observed and are compiled in Figure 2 and Table 2.

All tested detergents having an alkyl chain in their hydrophobic moiety showed high affinity for β-cyclodextrin (K_a in the range 10^4–10^5 M^{-1} as estimated from the relative CMC-shift, which in fact is the slope of the linear curves in Figure 2) as indicated by an almost equimolar CMC-shift (Figures 2A–2C). This group of detergents showed variable affinity for α-cyclodextrin ranging from high (nonylglucose; Figure 2A) to intermediate ($K_a \leqslant 5.10^3$ M^{-1}; dodecylmaltose, Figure 2B; Zwittergent 314; HECAMEG; Ammonyx LO) to not measurable (Triton X-100, Figure 2C). The detergents with a sterol-based hydrophobic moiety [CHAPS(O), cholate] displayed a completely different behaviour (Figure 2D). They showed high affinity for γ-cyclodextrin, but no measurable affinity for α- and β-cyclodextrin.

These results suggest that for every detergent the right cyclodextrin partner is available to produce an inclusion complex of sufficient stability to prohibit micelle formation. Whether this would also be sufficient to remove detergents from mixed micelles, was subsequently tested with a limited number of combinations (nonylglucose, DoM, Triton X-100 and Zwittergent 314 with β-cyclodextrin, CHAPS with γ-cyclodextrin).

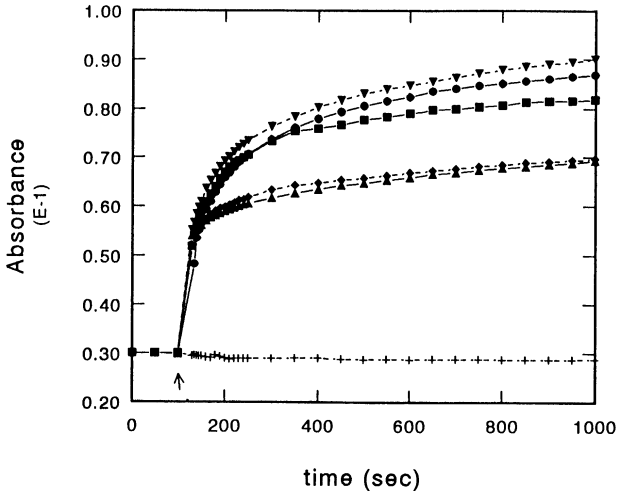

Figure 3 Turbidity development during cyclodextrin-mediated detergent extraction from mixed micelles

Photoreceptor membranes were dissolved in 20 mM detergent in buffer A (3 μM in Rh; lipid/protein ratio: 65/1) and any insoluble material was removed by centrifugation to yield a transparent solution (A_{700} 0.001). Solid HOM-β-cyclodextrin was added (arrow) to a final concentration of 20 mM. Turbidity development was then monitored as the increase in absorbance at 330 nm. Upon addition of cyclodextrin to 10 mM no change in absorbance was observed (+). Detergents shown: Nonylglucose (●), Zwittergent-314 (◆), dodecylmaltose (■), Triton X-100 (▼) and CHAPS (▲).

Cyclodextrins can selectively extract detergent from mixed micelles

All detergent–cyclodextrin combinations tested produced very similar results. Rhodopsin preparations with molar lipid to protein ratios from 50 to 300 were solubilized in 20 mM solutions of either detergent mentioned above. Occasional turbidity was removed by centrifugation so as to start with a transparent solution. Upon titration with the selected cyclodextrin no significant change was observed until the cyclodextrin concentration approached or slightly exceeded the detergent concentration. At that stage turbidity developed which can be visually observed or followed spectroscopically. Typical examples are given in Figure 3. It is clear that the rate and extent of the turbidity increase depends on the type of detergent. We have not yet found a clear correlation of these parameters with the final vesicle-size. Nevertheless, it is obvious that in all cases vesicles are produced, even with dodecylmaltose, a detergent with low CMC (0.16 mM), which is quite difficult to remove by hitherto available methods. A higher cyclodextrin/ratio (1.5 to 1) accelerates turbidity development, but does not change the final level (not shown). The time span over which the turbidity develops depends to some extent on the protein concentration and the lipid to protein ratio, but usually is in the order of 15–30 min. Only when appreciable 'contamination' with soluble protein is present, may the process take several hours to complete.

Several combinations, which did not produce a significant relative CMC-shift (DoM + γ-cyclodextrin, CHAPS + β-cyclodextrin) were also evaluated. Here, titration with cyclodextrin did not produce a significant increase in turbidity, until a large

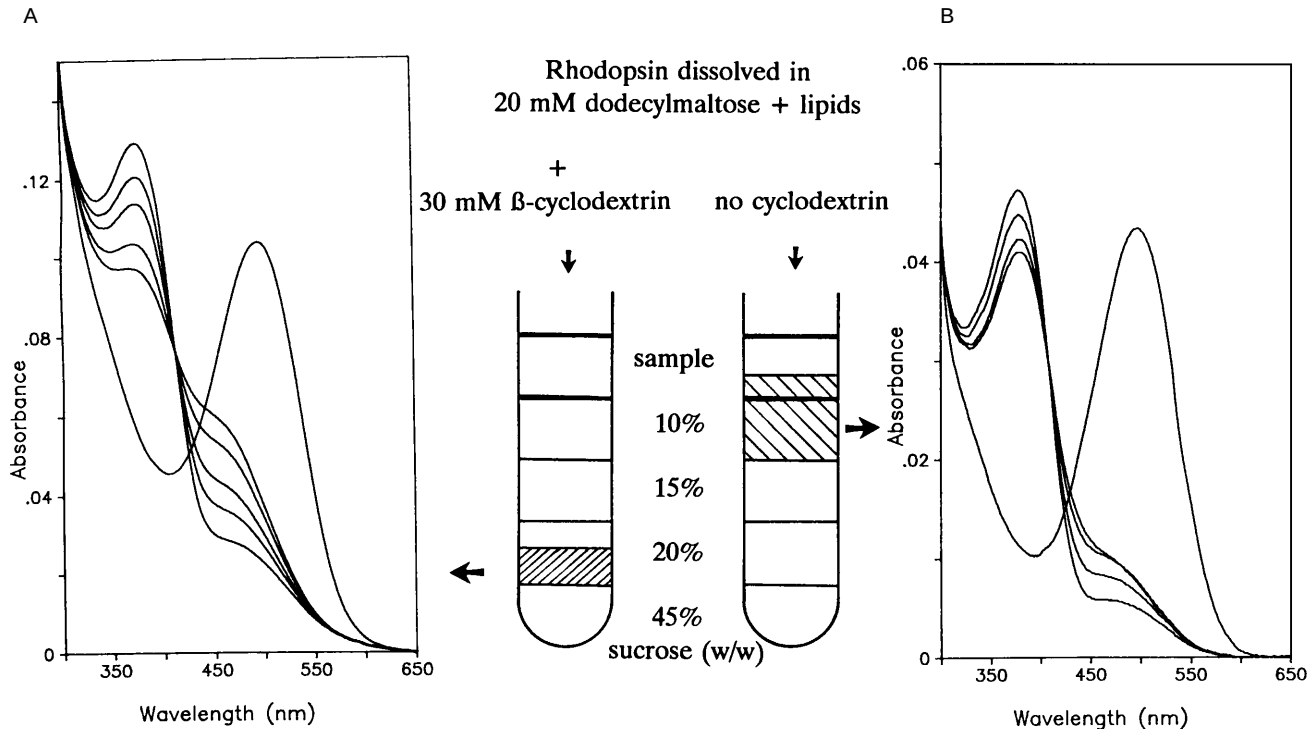

Figure 4 Schematic presentation of the decisive action of cyclodextrin in proteoliposome formation

Photoreceptor membranes were dissolved in 20 mM dodecylmaltose in buffer A (10 μM in Rh) and retina lipids were added to a final lipid/protein ratio of 150/1. Any insoluble material was removed by centrifugation. Half of the solution was not treated with cyclodextrin and layered directly on top of the step sucrose gradient shown on the right. After centrifugation, a transparent band containing detergent, lipid and protein was collected from the top layers of the gradient (hatched area). This material displayed a late photocascade pattern typical of detergent-solubilized rhodopsin (spectra at the right; cf. also Table 3). After prior treatment with 30 mM HOM-β-cyclodextrin a completely different pattern was observed, as shown at the left side. Following centrifugation a turbid proteoliposome band had accumulated onto the 20%/45% sucrose interface (hatched area). This material displayed the late photocascade pattern (spectra at the left) typical of rhodopsin in native photoreceptor-membranes (e.g. [39], Table 3). The detergent was again detected in the top layer and the 10% sucrose layer.

molar excess (5–10-fold) had been added. Under those conditions, however, low-affinity complex-formation with phospholipids can also occur, removal of the large amount of free and complexed cyclodextrin is complicated and only low yields of proteoliposomes are obtained. Hence, the relative CMC-shift has good predictive value. Detergent–cyclodextrin combinations which produce a nearly equimolar relative CMC-shift (i.e. $\geqslant 0.8$) can be successfully used for proteoliposome preparation.

Separation of proteoliposomes from inclusion complexes

To isolate the proteoliposomes several approaches were evaluated. Most simple and straightforward seemed to be velocity sedimentation by centrifugation. Although quantitative recovery of membrane protein could be achieved in this way, this approach was not satisfactory since complete separation of proteoliposomes from cyclodextrin complexes could not easily be realized and the functionality of proteoliposomes was often compromised.

Subsequently, we evaluated separation by dialysis, since the detergent–cyclodextrin complexes easily permeated through 30 kDa cut-off dialysis membrane, which under standard conditions completely blocks passage of protein-detergent micelles and proteoliposomes. Completely unexpected, however, we also encountered significant loss of protein with this approach. Control experiments then showed that haemoglobin, while completely retained by the dialysis membrane in the absence of cyclodextrins, rapidly passed through in the presence of these compounds. Apparently, cyclodextrins profoundly change the barrier properties of dialysis membrane. Although remarkable, we did not investigate this phenomenon further.

Finally, a combination of velocity and density centrifugation through a sucrose step-gradient proved successful. The decisive activity of the cyclodextrins in this procedure is schematized in Figure 4. In the absence of cyclodextrin, the protein remains bound to transparent mixed micelles, which distribute over the sample and top 10% sucrose layer. In the presence of cyclodextrin, the protein reconstitutes into a turbid proteoliposome band, which migrates through the density gradient until it encounters matching or higher density. A high-density (45% w/w) bottom layer was selected to prohibit pelleting of the proteoliposomes, since these pellets were not easily homogeneously resuspended. In addition, contamination by any aggregated protein could be avoided in this way, since these aggregates pass through the 45% sucrose layer to the bottom of the tube. TLC-analysis indicated that the detergent–cyclodextrin complexes largely stayed on top of the gradient. Cyclodextrin was included in the top layer of the gradient to trap any residual detergent travelling with the vesicles. The proteoliposomes migrated in the 20% layer or onto the 20%/45% interface, as schematically shown in the left panel of Figure 4. The presence of residual detergent or cyclodextrin in the proteoliposomes was analysed by Fourier-transform infrared spectroscopy, which can detect as little as 1 cyclodextrin molecule in 40 phospholipids (A. Pistorius, P. Bovee-Geurts and W. DeGrip, unpublished) and 1–2 detergent molecules in 20 phospholipids [6]. When the

Table 3 Characteristics of the late photocascade of rhodopsin preparations demonstrate efficacy of the cyclodextrin-extraction procedure*

Preparation†	% Meta I in Meta I–II equilibrium	Half-time Meta II decay (min)	Relative amount of Meta III produced ($\Delta A_{455}/\Delta A_{500}$)
ROS	15 ± 5	6.4 ± 0.7	0.36 ± 0.02
ROS-det	N.E.	1–5‡	< 0.1
Rh-protlip	18 ± 5	6.8 ± 0.5	0.39 ± 0.03

* The five detergents mentioned in the text were used to generate ROS-det and Rh-protlip. All detergents gave very similar results and the data were combined. The late photocascade was analysed as described under Experimental procedures and in [25]. Kinetic data were obtained by fitting an exponential curve through the data-points. Conditions: buffer A at 23 ± 1 °C. S.D. is given for $n = 11$–15.

† ROS, native photoreceptor membranes; ROS-det, detergent-solubilized photoreceptor membranes; Rh-protlip, rhodopsin reconstituted in retina-lipids using the cyclodextrin procedure; N.E., no equilibrium.

‡ Depends on detergent.

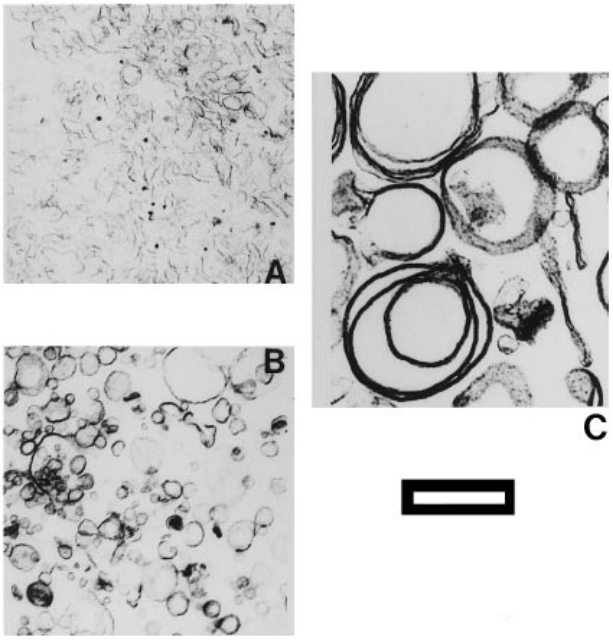

Figure 5 Ultrastructure of proteoliposomes produced by three different detergent–cyclodextrin combinations

(**A**), Dodecylmaltose + HOM-β-cyclodextrin; (**B**), Nonylglucose + HOM-β-cyclodextrin; (**C**), CHAPS + γ-cyclodextrin. The proteoliposomes were collected from the 20% sucrose layer or the 20/45% sucrose interface and prepared for electron microscopy as described under Experimental procedures. Bar represents 500 nm.

standard procedure was applied (cyclodextrin/detergent ratios between 1 and 1.5) the amount of detergent as well as of cyclodextrin in the proteoliposome preparation was below the detection level. Only at higher ratios (> 2.5) were residual amounts of cyclodextrin detected (up to 1 in 3 phospholipids). This explains why under those conditions the functionality of the proteoliposome preparations was often affected.

The recovery of membrane protein in the proteoliposome fraction ranged from 70 to 90%, higher loading resulting in better recoveries. The recovery of lipid depended on the lipid to protein ratio. At low molar ratios (50–150) recoveries of 60–90% were observed, which dropped to 50–60% at higher ratios (250–300). At ratios over 300, next to proteoliposomes protein-free liposomes were produced, which banded at the 0/10% interface.

All five detergent–cyclodextrin combinations tested gave very similar results, with the only caveat that recoveries with dodecylmaltose were usually on the low side of the ranges given above. While the most extensive studies were done with native rhodopsin, identical results were obtained with purified bacteriorhodopsin and recombinant rhodopsin as well as with less detergent-resistant membrane proteins (rhodopsin mutants, cone pigments). An additional advantage of the step-gradient procedure is that contamination with soluble proteins (up to 70% of total protein) is well tolerated and this fraction largely remains in the top layer of the gradient. Hence the procedure can simultaneously provide another 2–3-fold purification [39].

Functionality of proteoliposomes produced by detergent–cyclodextrin inclusion

Rhodopsin preparations, obtained with the five detergent–cyclodextrin combinations tested, were assayed for photochemical functionality using the last stages of the photoactivation cascade (Meta I $\rightleftarrows$ Meta II equilibrium, Meta II $\rightarrow$ Meta III transition). This part of the cascade is severely perturbed upon detergent solubilization: the Meta I $\rightleftarrows$ Meta II equilibrium is abolished and this transition fully proceeds to Meta II (λ_{max}: 380 nm); the decay of Meta II is accelerated and largely proceeds to free all-*trans* retinal (380 nm) instead of Meta III (455 nm). The latter pattern indeed was observed in the protein band after step-gradient centrifugation without treatment with cyclodextrin (Figure 4B). However, prior incubation with cyclodextrin restored the native pattern in the resulting proteoliposomes (Figure 4A; Table 3). All detergent–cyclodextrin combinations again gave very similar results.

In our experience, preparations of wild-type rhodopsin possessing a 'native' photoactivation cascade, also show a normal behaviour with respect to binding and activation of the G-protein, transducin. Nevertheless, this was incidentally checked for cyclodextrin-produced proteoliposomes using three different assays (the Meta I – Meta II shift assay measuring transducin-binding [29,30]; the transducin-fluorescence enhancement assay measuring release of activated T_α [31,32]; the cyclic GMP-pH shift assay, measuring activation of PDE by activated T_α [33]). In all assays the rhodopsin proteoliposomes showed similar kinetics and/or light sensitivity as native photoreceptor membranes (not shown).

Vesicle ultrastructure

The proteoliposomes produced with the combinations nonylglucose/β-cyclodextrin, dodecylmaltose/β-cyclodextrin and CHAPS/γ-cyclodextrin were analysed by electron microscopy (Figure 5). All combinations yielded mainly unilamellar vesicles with a smaller population of oligolamellar liposomal structures. The size range however varied considerably. Dodecylmaltose only produced small vesicular structures (diameter < 200 nm), nonylglucose a more variable population of intermediate size (100–400 nm), while CHAPS yielded relatively large vesicles (400–800 nm).

The orientation of rhodopsin in the proteoliposomes was investigated using limited proteolysis with proteinase K. This enzyme only clips rhodopsin at intracellular sites (C-terminal, loop i3) yielding final products with an apparent $M_r \leqslant 28$ kDa

according to SDS/PAGE analysis [37]. Since vesicles prepared from retina lipids are relatively leaky [35], penetration by enzyme was prohibited by using immobilized proteinase K. For our purpose, a lipid environment close to the native one was more important than its permeability properties, but of course lipids can be selected with other properties proper for any specific purpose. Short incubation of cyclodextrin-produced proteoliposomes with proteinase K resulted in proteolysis of 45–75% of rhodopsin present (not shown). Hence, as expected in view of their micellar origin, the produced proteoliposomes contain rhodopsin in a largely random orientation. However, cyclodextrin-extraction can equally well be combined with alternative reconstitution procedures aimed at more vectorial insertion of membrane proteins, e.g. using preformed vesicles [1,40].

DISCUSSION

Detergent–cyclodextrin interaction

Our results extend earlier reports on cyclodextrin–detergent interaction [18,41–44] to a large variety of polar head-groups and hydrophobic moieties. It is reasonable to assume that complex (CD) formation of detergents (D) and cyclodextrins (C) follows a simple equilibrium reaction ($C + D \rightleftarrows CD$) [38,45]. It can be easily estimated that when the cyclodextrin-induced CMC-shift is close to equimolar (that is $\geqslant 0.8$; 10 mM cyclodextrin producing a CMC-shift between 8 and 10 mM), the association constant K_a ($K_a = [CD]/[C] \times [D]$) is at least 10^4 M^{-1} and can be as high as 5×10^5 M^{-1}. Hence, such relative CMC-shifts indeed represent high-affinity interactions. These are observed for all alkyl-chain-containing detergents tested with β-cyclodextrin. Most representatives of this group show smaller relative CMC-shifts with α-cyclodextrin (0.3–0.6), corresponding to intermediate affinities ($1–5 \times 10^3$ M^{-1}). This agrees with the general trend for complex formation with α- and β-cyclodextrin [45]. We found that sterol-based detergents only had high affinity for γ-cyclodextrin. Probably their more bulky hydrophobic moiety is optimally accommodated only in the larger cavity of γ-cyclodextrin.

For several detergent–cyclodextrin combinations we do not observe a significant relative CMC-shift ($\leqslant 0.1$). This does not indicate that these combinations cannot form an inclusion complex. Low-affinity interactions (K_a in the range 10^2–10^3 M^{-1}), which are quite common for cyclodextrin inclusion complexes [12,13,45,46], cannot produce CMC-shifts > 0.2. The affinity of the detergent for a cyclodextrin species is apparently largely determined by the fit of its hydrophobic moiety within the cyclodextrin cavity. Thus, the bulky sterol group only allows an optimal fit with the large γ-cyclodextrin cavity. Likewise, the low affinity of Triton X-100 for α-cyclodextrin is explained by the presence of the more voluminous aromatic ring in its hydrophobic chain [47], in contrast to the other pure alkyl-chain detergents, which exhibit intermediate affinity for α-cyclodextrin. Apparently, size and charge of the detergent head-group contribute less to the detergent–cyclodextrin interaction, since a large variety of head-groups are well tolerated. This supports the concept that inclusion of the hydrophobic tail in the cyclodextrin cavity is the major driving force for complex formation [38].

Evidently, only high-affinity detergent–cyclodextrin complex formation can effectively compete with detergent association and inhibit micelle formation. The inverse process (detergent extraction from existing mixed micelles) is required for the generation of proteoliposomes. Since all processes involved are equilibria, however, the same relative affinities prevail in both conditions. Detergent extraction from mixed micelles probably proceeds via a two-stage process. First, detergent monomers, in equilibrium with micelle-associated detergent molecules, will be complexed by added cyclodextrin. The depleting monomer population will be replenished by release of detergent from the mixed micelles. Titration with cyclodextrin will continue to deplete the monomer population, thereby also depleting the mixed micelles, forcing them eventually to associate into a bilayer matrix. Since the driving force is detergent–cyclodextrin complexation and only a low selectivity exists for the detergent head-group, a detergent–cyclodextrin combination with sufficiently high affinity should be available for every detergent. Hence, we consider this approach to be of general applicability.

Properties of cyclodextrin-produced proteoliposomes

Following preparation of proteoliposomes, thorough separation from cyclodextrin is important to avoid eventual complications in subsequent functional or structural analyses due to random complex formation. This could be satisfactorily accomplished by sucrose step-density gradient centrifugation. This procedure has the additional advantage that it simultaneously removes contaminating aggregated or soluble protein. We tested the described cyclodextrin-extraction procedure on a small number of membrane proteins, and in all cases proteoliposomes with fully functional protein were obtained. In general, the essential condition for this, however, is to find a combination of detergent and lipid, which will sufficiently stabilize a membrane protein to allow considerable purification. Subsequent treatment with the proper cyclodextrin will then reproducibly produce functional proteoliposomes.

The size of the resulting vesicles can be manipulated by the choice of detergent. The use of dodecylmaltose resulted in a relatively small vesicle size (< 200 nm), which might explain the somewhat lower recoveries usually obtained with this detergent. CHAPS on the other hand gives rise to relatively large vesicles (400–800 nm), which could be interesting for special purposes (targeting, patch-clamp).

The procedure, as we applied it, starts with a homogeneous mixture of mixed micelles and is expected to generate proteoliposomes with a fairly random orientation of membrane protein in the lipid matrix. This is, indeed, what we essentially observe. Alternative procedures which would produce a less random distribution by vectorial insertion into preformed vesicles or via immobilized protein have been reported [1,7,40,48,49]. However, the essential final step in all procedures is removal of detergent. In these alternative procedures this can be equally reliably accomplished using cyclodextrin inclusion.

In conclusion, a novel procedure is described for the reconstitution of membrane protein into functional proteoliposomes using detergent extraction by means of inclusion complex formation with cyclodextrins. For all detergents tested a cyclodextrin partner could be found with sufficiently high affinity to perform this task. The procedure can be easily adapted to a variety of reconstitution methods and appears to be generally applicable, independent of type and CMC of the detergent.

We acknowledge Mr. Huib Croes for performing the electron microscopic analysis, Mr. Daniel Portier for the fluorometric T_α-release assay and Dr. Arthur Pistorius for the detergent and cyclodextrin analysis by FT-IR spectroscopy. This research was supported in part through the Space Research Organization of the Netherlands SRON (grant MG-038 to W.dG.).

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Received 2 June 1997/21 October 1997; 6 November 1997