The micelle to vesicle transition of lipids and detergents in the presence of a membrane protein: towards a rationale for 2D crystallization

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Received 23 November 1995; revised version received 5 February 1996

Abstract The assembly of two-dimensional membrane protein crystals in the presence of lipids was analyzed with quasielastic light scattering and electron microscopy. Mixtures of detergent-solubilized lipids and/or proteins were submitted to slow or rapid dilution while measuring the hydrodynamic radii of the aggregates. Lipids alone exhibited λ-shaped dilution curves with intermediate rod-shaped particles that converted into small vesicles. Depending on the protein-protein and protein-lipid interactions, detergent-solubilized protein-lipid mixtures showed a sharp transition from micelles to large, densely packed proteoliposomes. Electron microscopy revealed that formation of crystals occurred shortly after this phase transition.

Key words: Reconstitution; 2-dimensional Crystal; Porin OmpF; Human erythrocyte band 3; Photosystem I reaction center

1. Introduction

In spite of advances in X-ray techniques, and recent developments in NMR spectroscopy, the structure of most membrane proteins remains elusive. Of large diversity in function, these proteins share the feature of a hydrophobic belt that is required for their integration in the lipid bilayer. For structural analyses, membrane proteins need to be solubilized with detergents and purified. Solubilization tends to destabilize the proteins, particularly with the use of small detergents such as required for three dimensional (3D) crystallization. Therefore, detergent-solubilized proteins rarely form crystals suitable for X-ray analyses. A powerful alternative is the reconstitution of two dimensional (2D) membrane protein crystals in the presence of lipids. In this approach the native environment of membrane proteins is restored, as well as their biological activity [1]. Cryo-electron microscopy is then used to assess the 3D structure of the protein at atomic resolution [2,3].

Detergent-mediated reconstitution of membrane proteins is a frequently used method to obtain proteoliposomes for functional studies. Two mechanisms for protein insertion have been proposed [4–6]: (i) Detergent removal results in the formation of ternary micelles, which are converted to liposomes with homogeneously mixed components, and (ii) detergent-destabilized liposomes are formed prior to protein incorporation [4]. Which one of the two mechanisms applies in a certain case depends critically on the nature of the detergent used as well as on the rate of detergent removal [4], but is rather insensitive to the particular protein to be reconstituted [5]. In contrast, the self-assembling process leading to densely packed vesicles and 2D crystals involves protein-protein, lipid-protein, detergent-protein, and detergent-lipid interactions. Procedures described in the literature to obtain 2D crystals by reconstituting membrane proteins in lipid bilayers are often contradictory concerning the necessary prerequisites for the ingredient molecules and parameters (for reviews see [7,8]). This is understandable because the assembly process of membrane protein crystals in the presence of lipids is difficult to assess and is therefore not well understood.

In this report we describe an experimental approach that provides new insights into the formation of 2D membrane protein crystals. We measured the size of the ternary complexes in a mixture of proteins, lipids and detergent using quasi-elastic laser light scattering, a non-invasive method that is routinely used for the analysis of colloids. Comparing different proteins in such mixtures during dilution series revealed that the critical step in the crystallization process is the micelle to vesicle transition, a sharp λ-shaped transition previously observed in lipid-detergent systems by different techniques [9–13]. Dramatic differences of the particle size distributions during this transition proved to be indicative for crystallization, which in turn was assessed by negative stain electron microscopy.

2. Material and methods

2.1. Materials

Egg lecithin grade 1 was from Lipid Products, Nutfield, Surrey, UK; octylpolyoxyethylene (C_8E_{2-9}; 8-POE) was from Bachem, Bubendorf, Switzerland; and dodecyloctaoxyethylene ($C_{12}E_8$) was from Nikko Fine Chemicals, Tokyo, Japan; octyl-β-D-thioglucopyranoside (8-Thiogluc) was from Calbiochem, La Jolla, USA; and decylmaltoside (10-MALT) was from Sigma (St. Louis, USA). Lipids and detergents were used without further purification. All other chemicals were of analytical grade.

2.2. Proteins

E. coli B porin OmpF was extracted and purified from strain BZB3333 [14]. Human erythrocyte band 3 was solubilized and isolated following a modified protocol of Dolder et al. [15]. Briefly: Stripped erythrocyte membranes were solubilized with either 0.5% 10-MALT or 0.5% $C_{12}E_8$. Solubilized proteins were separated by centrifugation on 5–20% (w/v) sucrose gradients. Fractions containing band 3 (detected by SDS-polyacrylamide gel electrophoresis and immunoblot-

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**Martin Zulauf died in a tragic airplane accident in June, 1995.

Abbreviations: 8-POE, octylpolyoxyethylene; $C_{12}E_8$, dodecyl-octaoxyethylene; 8-Thiogluc, octyl-β-D-thioglucopyranoside; 10-MALT, decylmaltoside; CMC, critical micelle concentration; PBS, phosphate-buffered saline.

SSDI S0014-5793(96)00180-9

ting) were pooled and dialyzed against PBS containing an appropriate amount of either 10-MALT or $C_{12}E_8$. Photosystem I reaction centers from *Synechococcus* sp. (OD 24) were purified as described [16].

2.3. Mixed lipid-detergent micelles

Detergent-solubilized lipids were prepared by two methods, depending on whether the detergent is liquid (8-POE) or solid (the others). In the first case, lipids were dissolved in chloroform/methanol, dried in a rotatory evaporator and the lipid film was redissolved in phosphate-buffered saline (PBS: 150 mM NaCl, 10 mM sodium phosphate, pH 7.2) containing the appropriate amounts of the detergents. In the second case, the detergents were co-dissolved with the lipids in chloroform/methanol, dried and then rehydrated in PBS.

2.4. Light scattering

Quasi-elastic light-scattering experiments were performed at 90° scattering angle with the system ALV-3000 (ALV-Laservertriebsges m.b.H., Langen, Germany). Samples of about 0.6 ml were filtered through 0.2 μm polycarbonate membrane filters (Nucleopore, Sterico, Dietikon, Switzerland) into cylindrical quartz cells. Correlation functions were analyzed with the program CONTIN [17] which yields a distribution of relaxation times. Mean values for the diffusion coefficients D were calculated by taking the 2nd moment of the observed distribution. Hydrodynamic radii were calculated from the Stokes-Einstein equation: $R_h = kT/(6\pi\eta D)$, where k stands for Boltzmann's constant, T is the temperature and η the solvent viscosity. When the distribution of relaxation times was unimodal, its second moment was determined and the corresponding R_h was calculated. Near the phase transition the distribution was often multimodal, indicating size polydispersity and changing populations in the scattering volume. In these instances only the smallest and largest R_h values detected in the distributions were monitored.

2.5. Dilution series

Solutions of protein, lipid and detergent were prepared by mixing the appropriate detergent-solubilized components. These starting solutions correspond to dilution 1 in our terminology. Their exact composition differed depending on the experiment and is indicated in the legends to the figures. Solutions (typically 0.6 ml) were diluted by addition of detergent-free PBS in aliquots of 25 μl per min to different end concentrations ('slow' dilution). Alternatively, a defined volume of PBS was added to the starting solution by rapid mixing to obtain a specific end concentration ('rapid' dilution). Changes of particle sizes were measured by quasi-elastic light scattering, monitoring the amount of scattered light and the hydrodynamic radius R_h. All lecithin samples without protein could be filtered easily, even when aggregate sizes were large at the phase transition. By contrast, when lecithin and protein were present, very large aggregates formed that clogged the filter. In these instances, the filtering was omitted to obtain unbiased values for the radii.

During a dilution series the sizes of the aggregates changed because an increasing number of detergent molecules were removed from the aggregates to maintain the monomeric detergent concentration in the solvent at the critical micelle concentration (CMC [18]). The amount of detergent available for solubilization is the total amount of detergent minus its CMC. For example, the CMC of 8-POE in PBS at room temperature is 2.8 mg/ml (8 mM). Therefore, a solution with 12.5 mg/ml (35.7 mM) 8-POE contains 9.7 mg/ml (27.7 mM) solubilization-competent 8-POE ($M_r = 350$). Since a micelle ($R_h = 23 \pm 2$ Å, $M_r = 26250$) contains about 75 detergent monomers [19], the micelle molarity is 370 μM. Hence, we find on average 3.5 lecithin molecules per micelle in a solution containing 1 mg/ml (1.3 mM) lecithin ($M_r = 775$) and 12.5 mg/ml 8-POE. Upon dilution of this solution, the lipid and detergent concentration decrease. For example, at a dilution by a factor of 4, the lipid concentration is 0.25 mg/ml (325 μM), that of 8-POE is 3.125 mg/ml (8.9 mM) and the amount of solubilization-competent 8-POE is 0.325 mg/ml (900 μM), corresponding to a micelle molarity of 12 μM. Therefore, there are 27 lipid molecules in a micelle resulting in an increased R_h of the lipid-detergent complexes.

2.6. Electron microscopy

The sample solutions were applied to electron microscope grids covered with a hydrophilic carbon coated parlodion film. Grids were washed with two drops of water and stained with 0.75% uranyl formate. Samples were observed in a Hitachi H-7000 electron microscope, and images were recorded on Kodak SO-136 plates.

3. Results

3.1. Porin OmpF/lecithin in 8-POE

To compare the aggregation of lecithin and porin, two experiments were carried out, one with lecithin (1 mg/ml, 1.3 mM) dissolved in 8-POE (12.5 mg/ml, 35.7 mM), and the other with porin trimers (1 mg/ml, 9 μM) dissolved in the same amount of 8-POE. Both solutions were diluted to the end concentrations indicated in Fig. 1A. Lecithin (open circles, crosses) exhibits the λ-shaped dilution curve observed previously [13]. In the starting solution, particle radii were small (2.4 nm), corresponding to 8-POE micelles with 3–4 phospholipid molecules per micelle (see section 2). During the first dilution steps no significant increase of the sizes was observed. Between dilutions 4 and 5, where the detergent concentration was close to the CMC of 8-POE (indicated by the vertical line), the hydrodynamic radii increased rapidly as result of the micelle-to-rod transition [9,10] (see also section 4). Upon further dilution, these rod-like structures rearranged to vesicles with radii of 10 nm. The overall curve profile as well as the sizes of particles did not change significantly, irre-

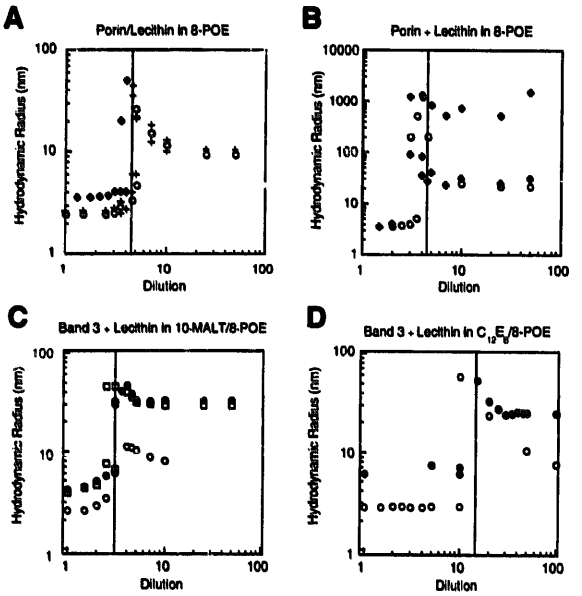

Fig. 1. (A–D) Hydrodynamic radii monitored by quasielastic light scattering as a function of the dilution factor. For multimodal distributions, only the smallest and largest R_h values are plotted. (A) Starting solutions were: lecithin 1 mg/ml in 12.5 mg/ml 8-POE (slow dilution: open circles; rapid dilution: crosses); 1 mg/ml porin in 12.5 mg/ml 8-POE (diamonds). (B) Dilution of 1 mg/ml porin + 1 mg/ml lecithin in 12.5 mg/ml 8-POE (diamonds: slow dilution, circles: rapid dilution). (C) Dilution of 0.23 mg/ml lecithin in 1.54 mg/ml 10-MALT/2.3 mg/ml 8-POE (open circles); 0.23 mg/ml band 3 + 0.173 mg/ml lecithin in 1.54 mg/ml 10-MALT/2.3 mg/ml 8-POE (filled circles); 0.23 mg/ml band 3 + 0.288 mg/ml lecithin in 1.54 mg/ml 10-MALT/2.3 mg/ml 8-POE (squares). (D) Dilution of 0.23 mg/ml lecithin in 0.77 mg/ml $C_{12}E_8$/2.3 mg/ml 8-POE (open circles); 0.23 mg/ml band 3 + 0.23 mg/ml lecithin in 0.77 mg/ml $C_{12}E_8$/2.3 mg/ml 8-POE (filled circles). The vertical lanes in A–D indicate the dilution at which the CMC is reached. For the detergent mixtures, the CMC was calculated according to Tanford [25].

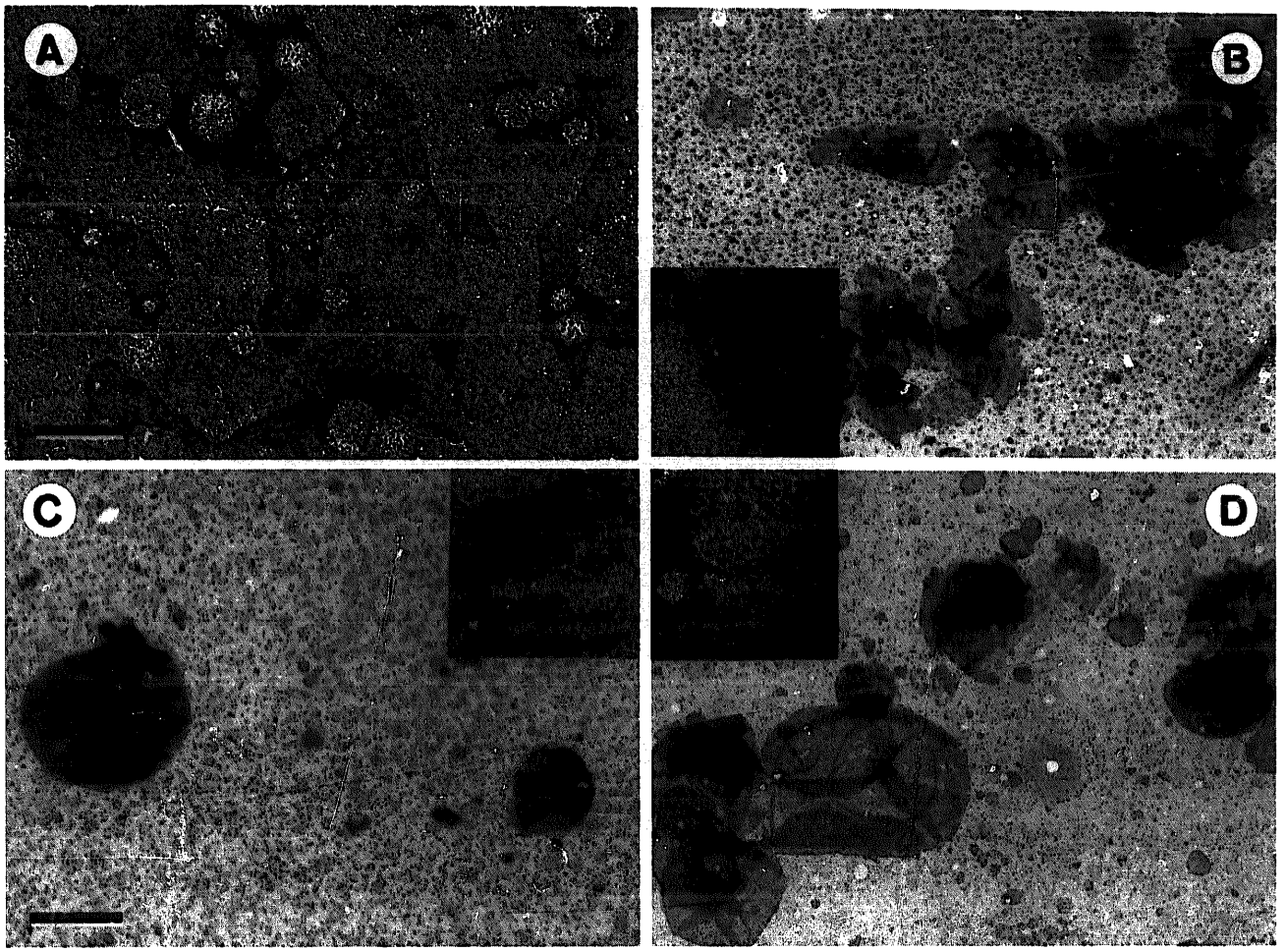

Fig. 2. Electron micrographs of negatively stained samples taken at different stages during the dilution of porin+lecithin in 8-POE (diamonds in Fig. 1B). (A) Dilution 2, (B) dilution 3, (C) dilution 5, (D) dilution 25. Insets in B–D: higher magnifications. Scale bars: 100 nm in A, 1 μm in B, insets at same magnification as A.

spective of whether the dilution was performed slowly (open circles) or rapidly (crosses; Fig. 1A).

In the starting solution of porin in 8-POE, a radius of about 3.3 nm was measured. This is a weighted average of the radius of empty micelles ($R_h = 2.3$ nm) and porin-detergent complexes ($R_h = 3.6$ nm, Zulauf, unpublished). Although there are more micelles than protein complexes, the latter dominate as result of their higher mass. Upon dilution (Fig. 1A, diamonds), the average radius increased slowly, indicating the reduction in the number of empty micelles and possibly the formation of a few larger protein oligomers. At the dilution 3.5, which corresponds to a micelle molarity of 30 μM, large protein aggregates coexisted with solubilized porin trimers. Upon further dilution, the size of the aggregates increased further, and at 4.5, when the CMC was reached, the protein precipitated massively.

3.2. Porin OmpF + lecithin in 8-POE (slow vs. rapid dilution)

The dilution behavior of a solution of porin and lecithin in 8-POE differs fundamentally from the two solutions of which it is the mixture (Fig. 1B, filled diamonds). In the starting solution up to dilution 2, sizes are dominated by the protein-detergent complexes ($R_h = 3.4$ nm). Formation of large complexes occurred already at dilution 3, where the detergent concentration was still above the CMC (marked with a vertical line), suggesting strong protein-protein and protein-lipid interactions. Although the number of large complexes may be small at dilution 3, they dominate the scattering. Sizes are very polydisperse, the largest having radii in the μm range. As displayed in Fig. 2, these complexes were true vesicles with diameters of up to several μm. While the vesicles appeared amorphous at dilution 3 due to the still high detergent concentration (Fig. 2B, inset), further slow dilution resulted in distinct vesicles showing crystalline packing of porin with increasing order (Fig. 2C,D, insets). If the starting solution was diluted in a single step to the end concentration, the size profile exhibited a λ-shape similar to that of lecithin, but the final hydrodynamic radius of the proteoliposomes was 25 nm (Fig. 1B, open circles).

3.3. Human erythrocyte band 3 + lecithin in 10-MALT/8-POE

The micelle-to-vesicle transition of lecithin in a mixture of decylmaltoside and 8-POE (molar ratio 1:2) followed a λ-shaped curve (Fig. 1C, open circles) similar to that of lecithin in pure 8-POE (Fig. 1A). In the presence of band 3 at LPR 0.75, a gradual increase of hydrodynamic radii was detected (Fig. 1C, filled circles), suggesting either protein-protein or protein-lipid complex formation. The main transition from

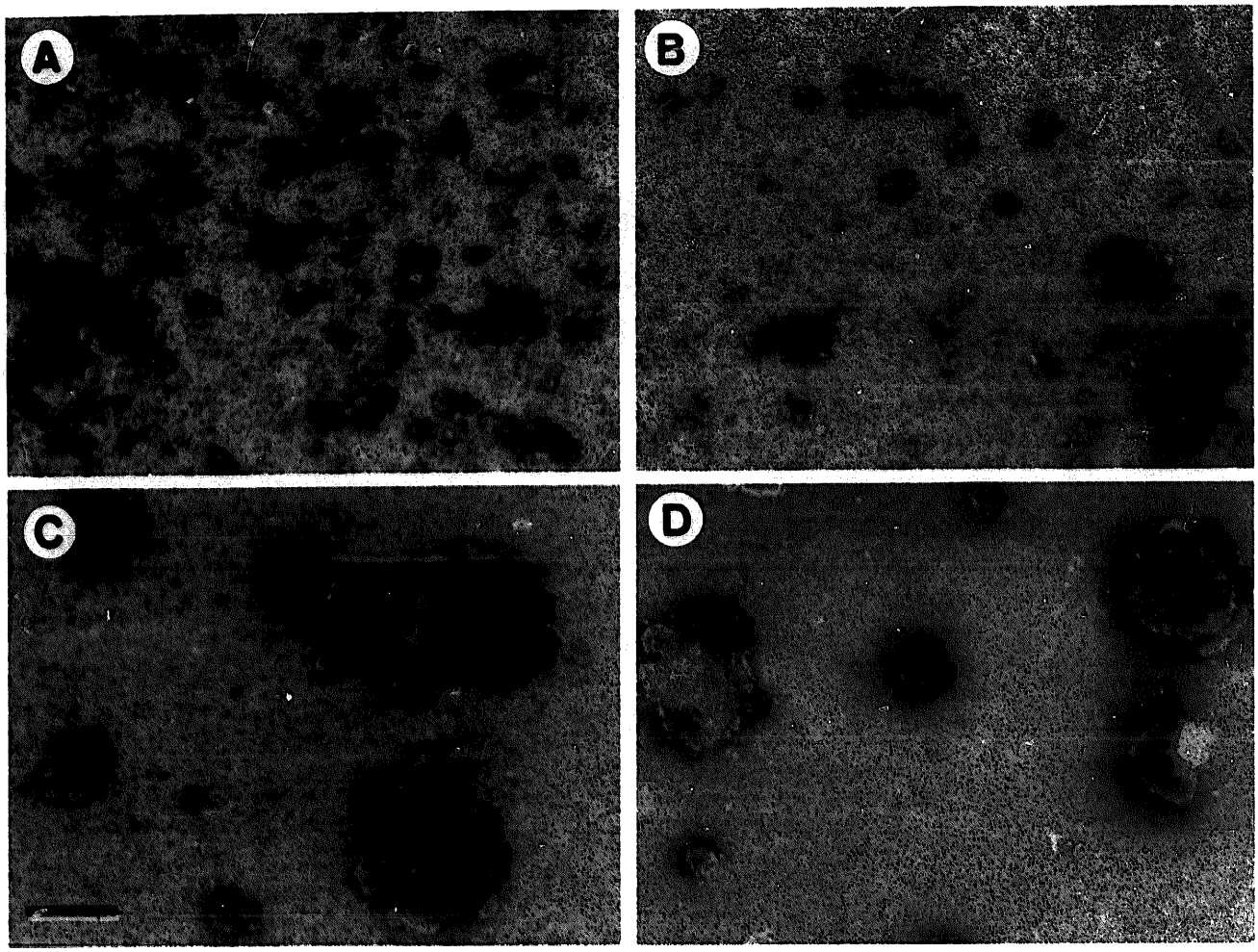

Fig. 3. Electron micrographs of negatively stained samples taken at different stages during the dilution of band 3+lecithin in $C_{12}E_8$/8-POE (filled circles in Fig. 1D). (A) Dilution 1, (B) dilution 5, (C) dilution 25, (D) dilution 25. Scale bar: 100 nm.

mixed micelles to vesicles occurred at the same dilution as for lecithin alone. At this dilution, the total detergent concentration was close to the CMC of the detergent mixture (3.32 mM, indicated by the vertical line). At higher LPR (1.25), the curve was slightly shifted towards lower dilutions (Fig. 1C, squares). Independent of the LPR, the radius of reconstituted vesicles stabilized at 30 nm. In contrast to the system porin+lecithin (see Fig. 1B), a significant horizontal shift of the curve towards lower dilutions in the presence of band 3 protein with respect to the lipid ground curve was not observed. This suggests only weak interactions between band 3 and lecithin.

3.4. *Human erythrocyte band 3 + lecithin in $C_{12}E_8$/8-POE*

The micelle-to-vesicle transition of lecithin dissolved in a mixture of $C_{12}E_8$ and 8-POE (molar ratio 1:4.6) followed a λ-shaped curve with an abrupt change of aggregate sizes at dilution 10 (Fig. 1D, open circles). In the presence of band 3, the mean size of particles did also not change up to this dilution (Fig. 1D, filled circles). In particular, there is no indication of protein-lipid or protein-protein complex formation prior to the main transition, such as documented in Fig. 1B. As is shown in Fig. 3, band 3 has been clearly incorporated into the vesicles, although the fast transition may suggest segregation of the protein and lipid to unspecific aggregates and empty vesicles, respectively. However, no 2D band 3 crystals were obtained by the dilution technique under both conditions.

3.5. *Photosystem I + lecithin in 8-Thiogluc*

The hydrodynamic radii of photosystem I and lecithin complexes in 8-Thiogluc (24 mM in the starting solution) could not be determined by light scattering. However, the transition from mixed micelles to vesicles was monitored by negative stain electron microscopy (Fig. 4). At dilution 4, corresponding to a detergent concentration still above the CMC of 8-Thiogluc (4 mM), large interconnected protein-lipid aggregates were observed clearly marked off from the background. At dilution 12, the first vesicle-like structures were detected with indications for regular packing of PS I reaction centers (Fig. 4B). Upon further removal of detergent, distinct vesicles with crystalline packing of PS I complexes were formed.

4. Discussion

The experiments described in the present study were de-

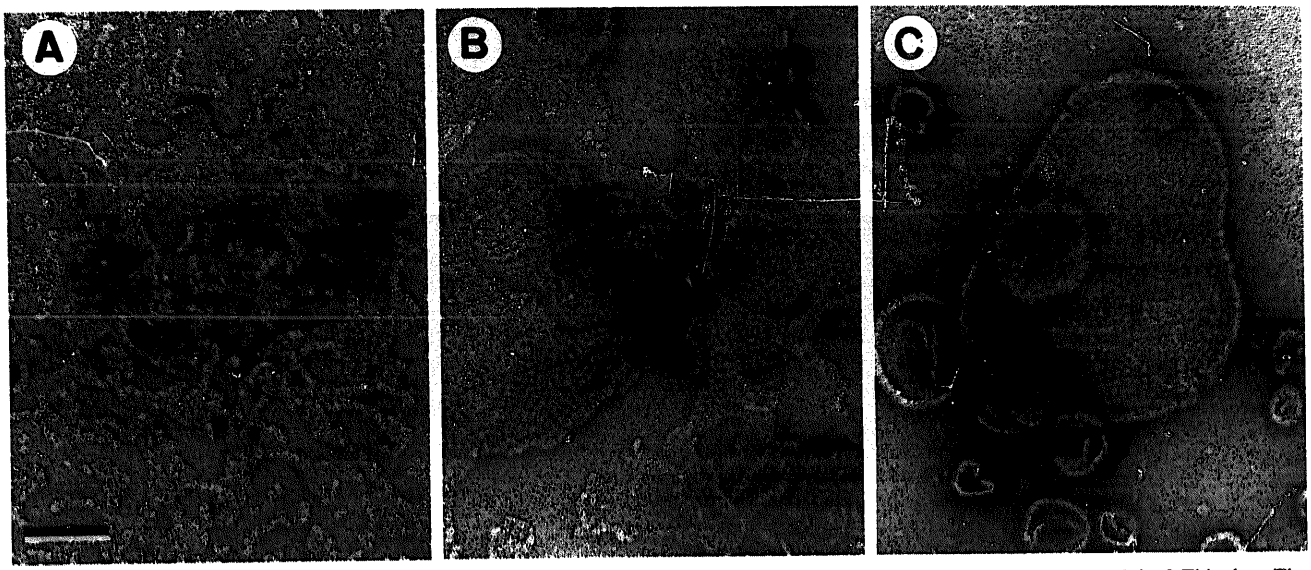

Fig. 4. Electron micrographs of negatively stained samples taken at different stages during the dilution of photosystem I in 8-Thiogluc. The starting solution contained 1 mg/ml PS I and 1 mg/ml lecithin in 7.5 mg/ml 8-Thiogluc. Dilution buffer was 10 mM MES pH 6, 100 mM NaCl, 10 mM $MgCl_2$. (A) Dilution 4, (B) dilution 12, (C) dilution 20. Scale bar: 100 nm.

signed to clarify the concept of reconstitution of membrane proteins via mixed micelles, a technique frequently used to prepare 2D crystals amenable to structure analysis by electron crystallography [7,8]. Three different membrane proteins were analyzed. Two of them (OmpF and PS I) pack readily into 2D crystals [13,16], while the third (band 3) is difficult to crystallize [15]. The assembly process was monitored during rapid and slow dilution series rather than during dialysis, because the latter excludes the study of low CMC detergents.

Our results show that the presence of a membrane protein alters the micelle-to-vesicle phase transition of a lipid and that this is the critical step in the assembly of 2D crystals. If the micelle-to-vesicle transition of lecithin is monitored by measuring the hydrodynamic radii of growing aggregates, a λ-shaped curve is obtained in all systems studied (lecithin in 8-POE, lecithin in 10-MALT/8-POE, lecithin in $C_{12}E_8$/8-POE). Such a curve is characteristic for micelle-to-vesicle transitions involving an intermediate rod-phase [11,12]. It also agrees with the ternary phase diagram for the system lecithin-sodium cholate-water, which shows a narrow extension of a rod-phase between the micellar and vesicular phase [20]. It has been shown that the process of liposome formation is in many respects the reverse of solubilization [21], the latter being described by a three-stage model [22]. Interestingly, in a number of studies on the solubilization of liposomes by detergents, a maximum of the turbidity was measured at the intermediate stage [23,24], similar to the maximum of the λ-curve in our experiments.

The presence of membrane proteins dramatically influences the shape of the dilution curve and therefore the micelle-to-vesicle transition. Our results suggest that the influence of membrane proteins on the phase transition depends on at least three different factors: (i) the speed of detergent removal; (ii) the lipid to protein ratio; and (iii) the hierarchy of interactions, viz. protein-protein, protein-lipid, protein-detergent and lipid-detergent. These factors are separately discussed below.

4.1. Speed of detergent removal

This factor was analyzed for the system lecithin in 8-POE and porin + lecithin in 8-POE (Fig. 1A,B). For lipids alone, the speed of dilution seems irrelevant. Neither the location of the λ-point nor the size of the final vesicles is affected. The intermediate rod-like aggregates that form at the micelle to vesicle transition are metastable and disassemble to form small vesicles. In the presence of porin, the results for slow dilutions are fundamentally different from the quick dilutions. When the large aggregates form, they are stable with respect to further slow dilution. This suggests that these aggregates are topologically different from rods and less prone to redissolution. Vesicles that incorporate protein would intuitively have this property. Indeed, inspection of the dilutions 3, 5 and 25 (Fig. 2) by negative stain EM reveals the presence of large vesicles containing porin in crystalline arrays. If the phase transition region is passed quickly by rapid dilution, the time for secondary aggregation of protein-lipid-detergent complexes present at the transition seems to be too short, resulting in small protein containing vesicles with radii of about 20–25 nm. These results indicate the importance of kinetic factors on the final proteoliposome sizes.

4.2. Lipid-to-protein ratio

Whereas for functional reconstitution of membrane proteins the LPR is normally kept high (≥ 10), it should be small for 2D crystallization. Densely packed vesicles can be obtained with a weight ratio of lipid to protein around 1:1 as used in our experiments. Accordingly, with all three proteins tested, vesicles that incorporate protein were packed at high density, an important prerequisite for proteins to establish lateral interactions necessary for 2D crystallization. The effect of the LPR on the dilution curve is only weak (Fig. 1C). Increasing the LPR causes a slight shift towards the left, indicating that the critical detergent-to-lipid (+protein) ratio at which the main transition occurs is reached earlier during a dilution series.

4.3. *Hierarchy of interactions, protein-lipid, protein-detergent and lipid-detergent*

For the system porin + lecithin in 8-POE (Fig. 1B) and photosystem I + lecithin in 8-Thiogluc (Fig. 4), we have observed that the formation of large protein-lipid-detergent complexes sets in at a dilution where the lipid alone would still be in the micellar state. We interpret this as follows: if protein-detergent and lipid-detergent complexes collide during the early stages of decreased detergent concentrations, they are stabilized due to strong protein-lipid interactions. Further dilution results in the formation of proteoliposomes and eventually in the rearrangement of the protein into a 2D lattice. From this we conclude that protein containing vesicles and 2D crystals are gradually formed during dilution. In particular, there is no indication for vesicle formation prior to protein incorporation. In addition, if protein-lipid interactions are strong, it is the protein that governs the phase transition. The result is a horizontal as well as a vertical shift of the dilution curve. In contrast, for the system band 3 + lecithin in 10-MALT/8-POE (LPR 0.75) and band 3 + lecithin in $C_{12}E_8$/8-POE (LPR 1) the micelle to vesicle transition coincides with that of the pure lipid. The absence of a horizontal shift of the dilution curve indicates only weak protein-lipid interactions. Thus, collisions between protein-detergent and lipid-detergent complexes are ineffective and do not give rise to larger aggregates prior to the main transition. As a consequence, no 2D crystals are formed. Nevertheless, the presence of the protein stabilizes the size of the final proteoliposomes, because there is no indication for an intermediate rod phase, suggesting that the phase transition is mainly governed by the protein.

Taken together, our results demonstrate that the micelle-to-vesicle transition of lipids and detergents shows basically two different characteristics, depending on the absence or presence of membrane proteins. In the presence of a protein at an LPR close to unity, the phase transition is mainly governed by the protein. From the horizontal as well as vertical shift of the measured transition curve, it is possible to qualitatively deduce the relative magnitudes of interactions between each pair of protein, lipid and detergent. The quality of reconstituted vesicles and 2D crystals probably depends on the hierarchy of these interactions, especially during the main phase transition of the system. If the protein and lipid segregate during this transition, there is no chance for the protein to incorporate into vesicles at a later step. We conclude that reconstitution of a membrane protein into phospholipid vesicles at high density to form 2D crystals requires the protein-protein and protein-lipid interactions to be higher than both the protein-detergent and lipid detergent interactions. Quasi-elastic light scattering allows these interactions to be monitored and opens new perspectives for the systematic optimization of crystallization conditions.

Acknowledgements: The authors are indebted to S. Karrasch for providing the purified photosystem I complexes, and to G. Tsiotis for helpful discussions. This work was supported by the Swiss National Foundation for Scientific Research (Grant 31-424335.94 to A.E.), and the Maurice E. Müller Foundation of Switzerland.

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