Detergent-Mediated Reconstitution of Membrane Proteins[†]

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ABSTRACT: The efficiency of reconstitution of the lactose transport protein (LacS) of *Streptococcus thermophilus* is markedly higher with Triton X-100 than with other detergents commonly employed to mediate the membrane insertion. To rationalize these differences, the lipid/detergent structures that are formed in the reconstitution process were studied by cryotransmission electron microscopy. Surprisingly, the two nonionic detergents Triton X-100 and *n*-dodecyl *β*-D-maltoside (DDM) affected the liposome structures in a completely different manner. Preformed liposomes titrated with Triton X-100 maintained their bilayer structure far beyond the onset of solubilization, and transport activity was maximal when LacS was inserted into these structures. With DDM the vesicular structures were already disrupted at the onset of solubilization and these membrane sheets were converted into long threadlike micelles at higher DDM to lipid ratios. Triton X-100 allowed the protein to be reconstituted with the hydrophilic surface exposed to the outside, whereas LacS was incorporated randomly when DDM was used. These differences in orientation are readily explained by the different lipid−detergent structures formed by Triton X-100 and DDM. The orientation of the reconstituted LacS protein is a critical factor for the activity of the protein as the kinetics of translocation is very different for opposite directions of transport.

Although many integral membrane proteins have been reconstituted into liposomes, the molecular mechanisms of membrane reconstitution are largely unknown and, generally, the efficacy of reconstitution is only qualitatively assessed. Structural data on the complexes formed by membrane proteins and detergents as well as the process of protein insertion into lipid bilayers is limited. Such information is crucial not only for the optimization of functional assays but also for the formation of ordered protein assemblies (2-D crystals) for structure analysis of the proteins by electron crystallography (*1*). We have used the purified lactose transport protein (LacS) of *Streptococcus thermophilus* as an example to follow the process of membrane reconstitution with different detergents, but the methodology appears to be applicable to other proteins as well (*2−4*). The LacS protein is a polytopic membrane protein that belongs to a large family of secondary transport proteins (*5*). It differs from most other secondary transport proteins by the presence of a hydrophilic carboxyl-terminal regulatory domain (*6*).

To optimize the reconstitution process for the purified LacS protein, the strategy of Rigaud et al. (*2, 7, 8*) was followed, which involves the stepwise solubilization of preformed liposomes and protein incorporation at the different stages of liposome solubilization. The physical state of the liposomes during the titration with detergent was followed by measuring the optical density at 540 nm. The solubilization curve can be divided into three stages (*9, 10*). During stage I, detergent molecules partition between the aqueous buffer and the bilayer. Stage II starts when liposomes are saturated with detergent (onset of solubilization $= R_{sat}$) and continues upon a further increase of the detergent concentration, thereby inducing liposome solubilization and the formation of micelles. At stage III, when the OD has reached its minimal value (R_{sol}), the mixture consists of micelles at varying detergent/lipid ratios. Although the optical density offers a convenient tool to follow the solubilization, the actual effects of the detergents on the liposomes are not revealed. Here, we describe the macromolecular structures that are formed by the lipid/detergent mixtures in the process of membrane reconstitution mediated by the nonionic detergents Triton X-100 and DDM. Based on these findings, a model is proposed for the unidirectional incorporation of LacS into liposomes mediated by Triton X-100 and for the random incorporation mediated by DDM.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *S. thermophilus* ST11($\Delta lacS$) carrying plasmid pGKHis was grown semi-anaerobically at 42 °C in Elliker broth supplemented with 0.5% beef extract and 20 mM lactose plus 5 μg/mL erythromycin (*8*).

Titrations of Liposomes with Detergent. Liposomes of acetone/ether-washed *Escherichia coli* lipids and L-α-phosphatidylcholine from egg yolk (Avanti Polar Lipids) in a ratio of 3:1 (w/w) were made by dissolving lipids (20 mg/mL) in 50 mM potassium phosphate, pH 7.0, plus 2 mM MgSO$_4$, followed by three freeze/thaw cycles and extrusion through polycarbonate filters with 400 nm pore size (*11*). Liposomes

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were diluted to 4 mg of PL[1]/mL and titrated by stepwise addition of ~0.5 mM Triton X-100 or DDM. The effect of the detergents on the liposomes was followed by measuring the optical density at 540 nm with a SLM-Aminco spectrophotometer (*9*).

Cryotransmission Electron Microscopy. A small droplet (ca. 5 μL) of (detergent-treated) liposome suspension was applied on one side of a Formvar film-coated (300 or 500 mesh) copper grid or to a holey carbon film on a 500 mesh copper grid; both sample preparations yielded identical results. The grid was mounted in a homemade plunging device, carefully blotted with filter paper, and immediately plunged into liquid propane cooled by liquid nitrogen. Specimen were subsequently stored under liquid nitrogen. The prepared frozen-hydrated specimens were analyzed in a Phillips CM10 transmission electron microscope equipped with a model 626 Gatan cryostage at a temperature of −160 to −170 °C. Images were recorded on a Kodak FGP film at 100 kV.

Purification and Reconstitution of LacS. The protein was purified essentially as described (*8*). For the reconstitution, preformed liposomes (4 mg of PL/mL) were equilibrated for 60 min with the appropriate amounts of DDM or Triton X-100, mixed with the purified protein in 200 mM imidazole, pH 7.0, 100 mM NaCl, and 10% (v/v) glycerol, plus 0.05% detergent, and subsequently, the mixture was incubated for 30 min at 20 °C under gentle agitation. The final protein-to-lipid ratio was always 1:100−150 (w/w), and the final glycerol concentration never exceeded 1% (v/v). Polystyrene beads (Bio-Beads SM2, Bio-Rad) were added at a wet weight of 80 mg/mL and the samples were incubated for another 2 h at room temperature to remove the detergent. Fresh Bio-Beads were added twice and the samples were incubated at 4 °C for 3 h and overnight, respectively. The proteoliposomes were washed with 50 mM potassium phosphate, pH 7.0, harvested by centrifugation, resuspended in 50 mM potassium phosphate, pH 7.0, and 2 mM MgSO$_4$ plus varying concentrations of lactose (see figure legends), and stored in liquid nitrogen.

Lactose Counterflow in Proteoliposomes. After the samples were thawed at room temperature, the liposomes were extruded through 400 nm polycarbonate filters. In a number of experiments, the proteoliposomes were used directly after the removal of detergent by Bio-Beads, thereby avoiding the freeze/thawing step; we never observed significant differences in activity or orientation of the protein with or without the freeze/thawing step (not shown). After centrifugation, aliquots of concentrated proteoliposome suspension were diluted into 200 μL of potassium phosphate, pH 7.0, and 2 mM MgSO$_4$ (KPM) plus [^{14}C]lactose at concentrations indicated. The transport reactions were performed at 30 °C and processed as described previously (*8*).

Efficiency of Protein Reconstitution. To determine the efficiency of protein reconstitution, LacS was labeled with the photoactivatable reagent [^{125}I]TID (370 GBq/mmol).

Membrane vesicles (4 mg of protein/mL) were incubated with 65 μM TID and the probe was cross-linked by incubating the samples under UV light (254 nm) for 2 min at 0 °C. Subsequently, the membrane vesicles were solubilized and LacS was purified and reconstituted as described. The proteoliposomes were separated from nonincorporated protein by sucrose gradient centrifugation (*12*). To visualize the proteoliposomes in the gradient, octadecylrhodamine β-chloride (R$_{18}$) was incorporated at 50 nmol/mL of liposomes (20 mg of PL/mL) prior to the freeze/thaw/extrusion cycles. Nonincorporated R$_{18}$ was removed by washing with 50 mM potassium phosphate, pH 7.0. Fractions from the sucrose gradient containing lipid were pooled and the amount of protein associated with the lipid was compared to the total amount of protein used for the reconstitution.

Orientation of LacS in Proteoliposomes. The orientation was determined with antibodies raised against the C-terminal part of the protein, i.e., the cytoplasmically located IIA domain. (Proteo)liposomes were washed twice with 0.5% BSA in PBS and resuspended at 50 μg of LacS/mL. Subsequently, they were incubated for 2 h with antibodies raised against the purified IIA domain of LacS (Gunnewijk and Poolman, unpublished results) or preimmune serum at a 1000-fold dilution. After the proteoliposomes were washed 4 times with 0.5% BSA in PBS, the proteins were separated by SDS−PAGE. The proteins were blotted onto poly(vinylidene difluoride) membranes and the amount of bound antibody was determined by densitometry, following detection with a secondary antibody−alkaline phosphatase conjugate using CSPD as a substrate.

RESULTS

Lactose Transport Activity. To optimize the detergent/lipid ratio for reconstitution, liposomes were titrated stepwise with DDM. The solubilization of the liposomes was followed by measuring the OD$_{540}$, and after equilibration with the appropriate amount of detergent, LacS was added at a 1:100 protein-to-lipid ratio and reconstituted as described. The dependence of the transport activity on the DDM concentration, i.e., the physical state of the liposomes, is shown in Figure 1. In case of DDM, transport activity was only obtained when liposomes were destabilized with a certain minimal concentration of detergent (>3.5 mM). This coincides with the maximum optical density in the titration curve, which is indicative for the onset of solubilization (R_{sat}; detergent-to-lipid ratio ~ 1). The transport activity was highest at R_{sat}, and when the detergent/lipid ratios were increased further the transport activity declined. At the highest DDM concentrations used (R_{sol}; detergent/lipid ~ 3), the activity had dropped more than 5-fold.

Also in the case of Triton X-100, the liposomes used for the reconstitution required a minimal detergent concentration for activity (Figure 1, inset). Importantly, the Triton X-100-mediated reconstitutions yielded transport activities that were relatively constant over a broad detergent concentration range, and these activities were always higher than those of DDM-mediated reconstitution. An explanation for the observed differences might be found in the way these detergents interact with the liposomes and affect the macromolecular lipid/detergent structures.

Cryotransmission Electron Microscopy of Liposomes Titrated with Triton X-100 or DDM. Cryo-TEM pictures were

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[1] Abbreviations: BSA, bovine serum albumin; TEM, transmission electron microscopy; CSPD, disodium 3-(4-methoxyspiro{1,2,-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1]decan}-4-yl)phenyl phosphate; DDM, *n*-dodecyl β-D-maltoside; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PL, phospholipids; SDS, sodium dodecyl sulfate; TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; Triton X-100; poly(ethylene glycol) *p*-isooctylphenyl ether.
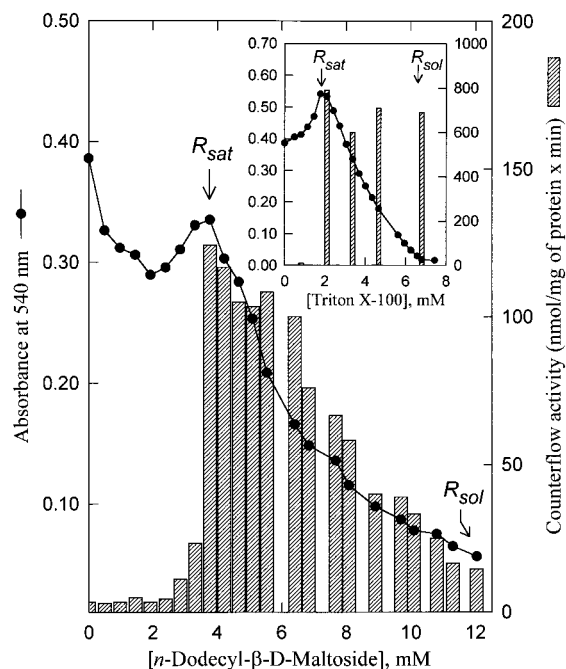
FIGURE 1: Lactose counterflow activity in proteoliposomes made via DDM- or Triton X-100-mediated reconstitution. Purified LacS protein was added to preformed liposomes (4 mg of PL/mL) equilibrated with different concentrations of DDM or Triton X-100. The physical state of the liposomes was followed by measuring the OD_{540} (●). After removal of detergent, the proteoliposomes were washed and resuspended into potassium phosphate, pH 7.0, and 2 mM $MgSO_4$ (KPM) plus 10 mM lactose. The counterflow reaction was started by diluting concentrated proteoliposome suspensions 200-fold into KPM containing 54 μM [^{14}C]lactose, final concentration.
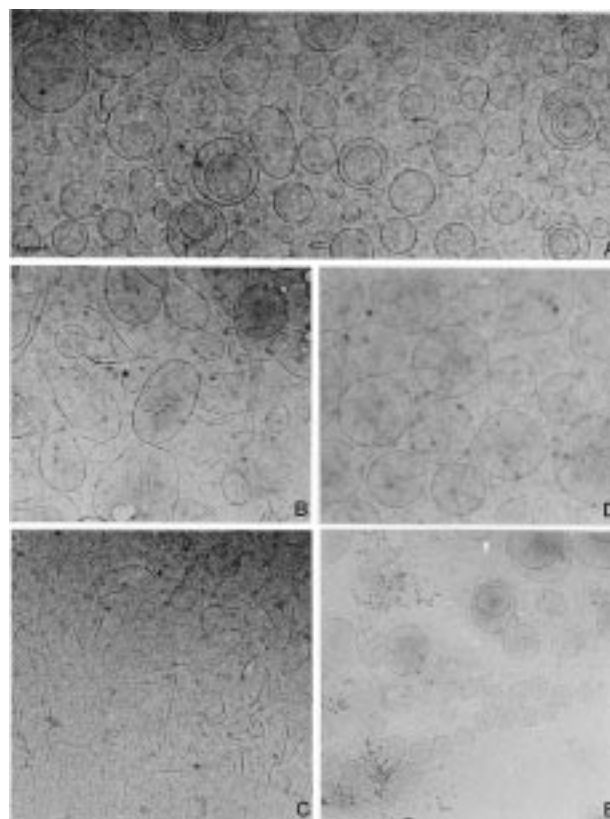

FIGURE 2: Cryotransmission electron micrographs of liposomes titrated with DDM or Triton X-100. Liposomes were diluted to 4 mg/mL and titrated with DDM or Triton X-100. Shown are preformed liposomes without detergent (A) and liposomes treated with 3.8 mM DDM (R_{sat}) (B), 5.7 mM DDM (C), 1.8 mM Triton X-100 (R_{sat}) (D), or 2.7 mM Triton X-100 (E). Bar represents 100 nm.

taken of intact liposomes after extrusion through 400 nm filters. In general, the liposomes were unilamellar and regularly shaped, and >90% had a diameter of 100−300 nm (Figure 2A). Equilibration of the liposomes with Triton X-100 at R_{sat} did not result in observable differences in the macromolecular structures as assessed by cryo-TEM, whereas the OD_{540} did increase (Figure 2D). At these detergent/lipid ratios, the initial increase in OD probably reflects a swelling of the liposomes due to partitioning of detergent molecules into the lipid bilayers and/or fusion of the liposomes. Surprisingly, a significant fraction of the liposomes remained intact at stage II of the solubilization, even at Triton X-100 concentrations that are far beyond R_{sat} (Figure 2E). The decrease in OD_{540} corresponds with a decrease in the size of the liposomes. It seems that, during the solubilization, lipids are extracted by the detergent without disruption of the macromolecular structure of the liposomes. Only at concentrations above R_{sol} (7 mM) were liposomes not observed any longer, and all the lipid seemed to be present in mixed micelles of lipid and detergent. However, even at relatively high detergent/lipid ratios, the lipids still tended to form liposomal structures despite the relatively high concentration of Triton X-100 in the bilayers.

These observations are in sharp contrast to the results obtained with liposomes titrated with DDM. Starting with the same liposome suspension, the R_{sat} was reached at higher detergent concentrations (4 mM) and the equilibration of DDM with the lipids was slow (2−3 h). Cryotransmission electron microscopy showed that only a few liposomes were

still intact at this point. Aberrantly shaped structures of lipid sheets and open liposomes predominated under these conditions (Figure 2B). Higher concentrations of DDM led to a further rearrangement of the lipids, and long threads (>200 nm), mimicking stretched micelles of lipid and detergent (see Discussion), were formed during stage II. Intact liposomes or even sheets of lipids were barely detectable under these conditions (Figure 2C). The rearrangement of liposomes into these unique structures is probably slow and explains the, initially, slow equilibration of DDM and lipid. In stage II the OD_{540} decreased further and equilibration became more rapid (reached within a few minutes), and the electron microscopic pictures showed that the threads became smaller. Ultimately, at concentrations above R_{sol}, all the lipid and DDM was present in micelles.

Efficiency of Protein Reconstitution. To assess the efficiency of reconstitution into the different structures formed during the solubilization of the liposomes with Triton X-100 and DDM, the amount of radiolabeled LacS associated with liposomes was determined after fractionation of the protein and (proteo)liposomes on sucrose gradients. The reconstitution with Triton X-100 was very efficient and at least 95% of the protein was incorporated when different concentrations of Triton X-100 were used to mediate the reconstitution. For DDM the efficiency of reconstitution at R_{sat} and R_{sol} were 85% and 80%, respectively, but this does not explain the lower transport activities observed. At concentrations of DDM above R_{sol}, the reconstitution was less reproducible
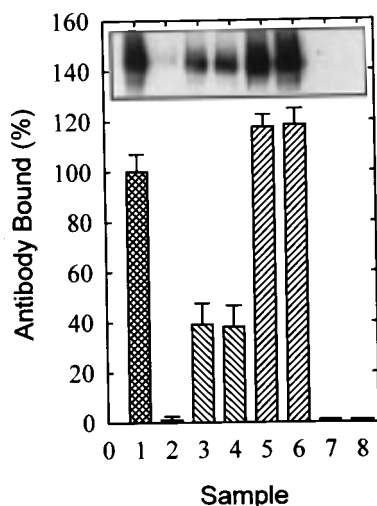
FIGURE 3: Orientation of LacS in proteoliposomes. Proteoliposomes were resuspended at 50 μg of LacS/mL in PBS plus 0.5% BSA and, subsequently, incubated for 2 h with antibodies raised against the IIA domain of LacS (lanes 1−6) or preimmune serum (lanes 7 and 8). Shown are Immune-precipitated LacS, set at 100% (lane 1); liposomes (lane 2); proteoliposomes made with 4 mM DDM (lane 3); 8 mM DDM (lane 4); 1.8 mM Triton X-100 (5); 4 mM Triton X-100 (lane 6); 4 mM DDM (lane 7); and 1.8 mM Triton X-100 (lane 8).

and the fraction of protein inserted into the proteoliposomes diminished in some cases to ∼50%.

Orientation of LacS in Proteoliposomes. The orientation of the incorporated LacS protein was determined for reconstitutions at DDM and Triton X-100 concentrations that gave the typical macromolecular lipid−detergent structures as shown in Figure 2 (B,C and D,E, respectively). The binding of antibodies specifically recognizing the hydrophilic IIA domain of LacS was compared at R_{sat} and R_{sol} of DDM and Triton X-100 (Figure 3). In case of Triton X-100-mediated reconstitution, the antibody binding was maximal and equivalent to total immune-precipitated protein used for the reconstitution at both detergent concentrations. Since all the IIA domain was available for the antibodies, the LacS protein must be reconstituted in an inside-out orientation. About 35% of the maximal amount of antibody bound, when the reconstitution was performed at DDM concentrations equivalent to R_{sat} and R_{sol} (Figure 3, lanes 3 and 4). The low amount of antibody binding cannot only be explained by a lower amount of LacS in the proteoliposomes as the reconstitution efficiency was only 10−15% lower for DDM. We conclude that LacS incorporates randomly into liposomes when DDM is used to mediate the reconstitution, whereas the incorporation is unidirectional (inside-out) when Triton X-100 is used.

Kinetic Parameters of Lactose Transport. Although secondary transport proteins generally operate reversibly in the two opposing directions of transport, i.e., the direction of transport is determined by the directionality of the driving force and not by the orientation of the protein (*13*), the kinetic parameters can be different for outside to inside and inside to outside translocation. The apparent affinity constant (K_m^{app}) for lactose self-exchange at the outer surface of the membrane in vivo is about 10 mM (*14*). Since Triton X-100-mediated reconstitution results in an inside-out orientation of the LacS protein, the counterflow (exchange) activity should not saturate up to an internal lactose con-
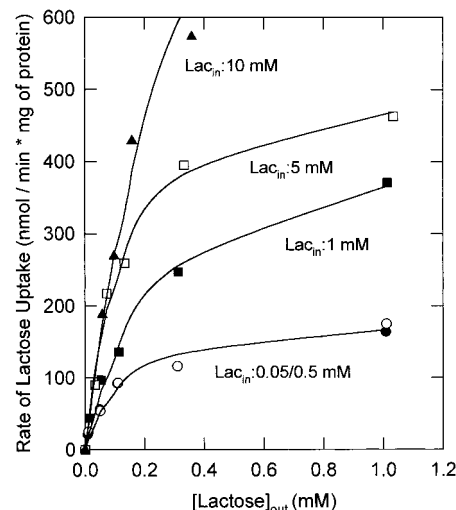

FIGURE 4: Kinetics of lactose counterflow. Proteoliposomes made by Triton X-100 (2 mM) mediated reconstitution, were equilibrated with different concentrations of lactose and subsequently diluted 200-fold to start the transport reaction at the [^{14}C]lactose concentrations indicated. Initial uptake rates were determined in triplicate, and the averaged data were fitted to the Michaelis−Menten equation.

centration of 10 mM. To study the kinetic parameters of LacS-mediated counterflow transport, the internal and external lactose concentrations were varied over a wide range. The data clearly show that upon increasing the outside lactose concentration the uptake saturated with K_m^{app} values in the submillimolar range, whereas the uptake rate increased almost linearly with internal lactose concentrations up to 10 mM (Figure 4). The latter value is consistent with the low-affinity site at the outer surface that was reported in the in vivo studies. The K_m^{app} in the submillimolar range, i.e., 0.1−0.3 mM, determined for the outer surface of the membrane in the proteoliposomes must therefore reflect the internal surface in vivo.

DISCUSSION

In this paper, the interactions of DDM and Triton X-100 with liposomes made of *E. coli* lipids and egg PC were studied by cryotransmission electron microscopy, and the possible mechanisms of reconstitution of a membrane protein in the formed macromolecular structures are described. From turbidity measurements, it was already clear that three stages can be distinguished during the process of membrane solubilization (*2, 9*). First, the detergent incorporates into the lipid bilayers (stage I), which results in a peak of the turbidity at the onset of solubilization (R_{sat}). Subsequently, the liposomes disintegrate (stage II) and a transition takes place from bilayer structures to mixed micelles. Finally, at R_{sol}, the liposomes are completely solubilized (stage III) and further changes only involve the ratio of detergent and lipids in the micelles. Although the turbidity measurements offer a good method to follow the solubilization process, they do not give any information on the exact mechanism and the structures formed during the solubilization. By employing cryo-TEM on the lipid−detergent mixtures, we now demonstrate that the second stage in the solubilization is completely different for Triton X-100 and DDM. The implications for protein reconstitution are schematically depicted in Figure 5 and are explained below.
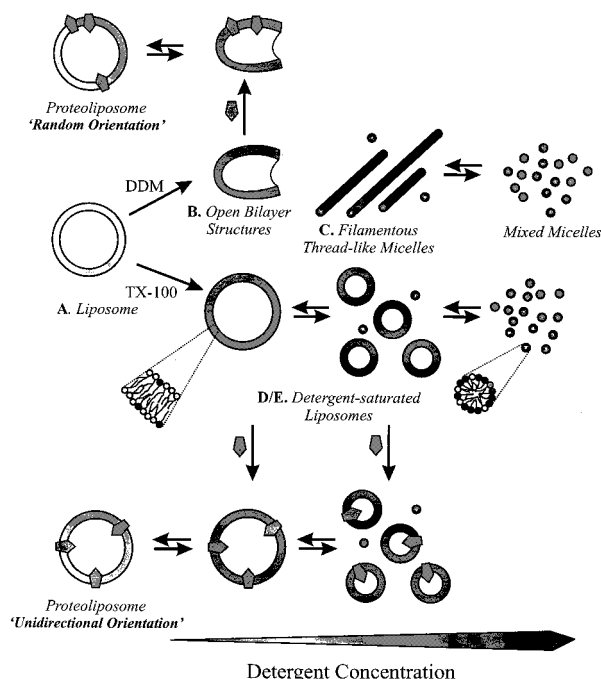
FIGURE 5: Model for membrane protein reconstitution mediated by DDM or Triton X-100. A−E correspond to the structures observed by cryo-TEM (Figure 2). Pentagons represent LacS protein.

In case of Triton X-100, intact liposomes were observed even near the end of stage II. An equilibrium exists between Triton X-100-saturated liposomes and mixed micelles that diffuse in and out of the bilayer (Figure 5). When more Triton X-100 is added, the equilibrium shifts from the liposomal toward the micellar state, and the opposite occurs when Bio-Beads are used to lower the detergent concentration. The decrease in OD_{540} with increasing Triton X-100 concentrations parallels a decrease in the diameter of the liposomes, but the bilayer structure remains intact. We speculate that Triton X-100 has a molecular shape that stabilizes membranes, in particular those that are largely composed of nonbilayer lipids such as PE and cardiolipin (see also ref *15*). Notice that the *E. coli* lipid/egg PC mixture used consists of about 50% PE and 10% cardiolipin. Patches of Triton X-100 molecules enable protein molecules, surrounded by detergent, to diffuse from the solution into the membrane. By removal of Triton X-100 with the hydrophobic beads, the equilibrium is shifted toward protein insertion. At the highest concentrations of detergent, when only mixed micelles are present, the addition of the polystyrene beads will (quickly) drive the formation of bilayers into which the protein will insert. Membrane proteins such as LacS, of which the inner and outer surface differ significantly in hydrophobicity, will insert unidirectionally as it is unfavorable for the hydrophilic (IIA-) domain to penetrate the lipid bilayer once a closed vesicular structure is formed. Moreover, the liposomes initially formed from the mixed micelles are small in diameter, which induces a strong curvature of the membrane and a lipid asymmetry that might favor the unidirectional insertion even more (*16*).

With DDM, liposomal structures are only sustained until the onset of solubilization (R_{sat}). At this point a large fraction of the liposomes have lost their integrity and are present as membrane sheets. The highest transport activities were obtained when LacS was mixed with these structures. It seems likely that membrane proteins insert into these structures from both surfaces, without the need of the hydrophilic IIA domain to cross the bilayer, leading to a random orientation. DDM is characterized by an hydrophobic tail of intermediate length and a bulky hydrophilic headgroup (*17*). The alignment of these molecules at saturating concentrations will cause curvature of the membrane and disruption of the bilayer structure. Unlike Triton X-100, the physical properties of DDM support the formation of long micellar threads at intermediate detergent/lipid ratios (stage II). While this work was in progress, threadlike structures were also reported for liposomes composed of egg phosphatidylcholine and egg phosphatidic acid that were treated with DDM (*18*). Similar threads, with about the thickness of a phospholipid bilayer, have also been described for mixtures of octyl glucoside and phospholipids (*19*). It is hard to imagine that a membrane protein will incorporate into such threads since they do not seem to represent bilayer structures but rather form micellar structures (Figure 5). We propose that, with DDM to mediate the reconstitution, membrane proteins are only incorporated into the membrane sheets that are present at R_{sat}, i.e., when the stretched micelles are converted into liposomes. It is obvious that the characteristics of DDM and Triton X-100 are also relevant for 2-D crystallization of membrane proteins. For stable arrays of protein and lipids, Triton X-100 may be a much more suitable detergent than DDM as it permits protein insertion over a much broader range of detergent-to-lipid ratios and, consequently, exposes the protein for shorter periods to conditions that may lead to inactivation and/or aggregation.

The more or less random orientation of LacS in proteoliposomes obtained with DDM also explains in part the lower transport activity measured in the counterflow assay. For proteoliposomes obtained via Triton X-100-mediated reconstitution, the affinity constant for lactose at the outer surface of the membrane was ~0.2 mM, whereas it was at least 10 mM at the inner surface. In the case of the DDM-mediated reconstitution about 50% of the molecules have the low-affinity side exposed to the outside of the proteoliposomes. Thus, at a substrate concentration of 54 μM, about half of the molecules will not contribute significantly to the overall transport activity. The difference in transport activity at "low" and "high" concentrations of DDM reflects, in our opinion, loss of lipid, which is more pronounced for higher concentrations of DDM. The activity of LacS appears to depend strongly on the final lipid/protein ratio (unpublished results) and will thus be lower when more lipid is lost.

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