Chapter 7

DETERGENT-MEDIATED MEMBRANE PROTEIN RECONSTITUTION

Jan Knol, Robert H.E. Friesen, and Bert Poolman

INTRODUCTION

Organisms from all kingdoms of life need to maintain their cytoplasmic contents within narrow limits (homeostasis of pH, ion concentrations, osmolarity, etc.). In particular microorganisms (bacteria, archaea and lower eukaryotes) are confronted with relatively dramatic variations in the environment, and the corresponding homeostatic mechanisms thus need to be active and well-regulated. The cytoplasmic membrane harbors most of the essential components for these processes as well as the systems that generate and/or interconvert metabolic energy, transport nutrients into the cell, and transport metabolic end-products into the medium (Gennis, 1989; Poolman and Konings, 1993; Paulsen *et al.*, 1998).

Membranes mainly consist of lipids and proteins, and their relative amounts can vary significantly. For example, the lipid/protein (w/w) ratio in human myelin membranes is 3-4 and in the purple membranes of *Halobacterium salinarium* it is 0.2 (Devaux and Seigneuret, 1985). The cytoplasmic membrane of *Escherichia coli* has an average lipid to protein ratio of about 0.4 (w/w) with the major lipid components being: phosphatidylethanolamine (PE,~74%), phosphatidylglycerol (PG, 5 to 19%) and cardiolipin (CL, 3 to 20%). With an average protein molecular weight of 50,000 and only phospholipids present, this would mean a molar lipid to protein ratio of about 50. If a membrane protein is represented as a cylinder with a radius of 18 Å, it would take about 32 lipid molecules to completely surround the protein (Gennis, 1989). This would mean that more than 50% of the lipid molecules is in contact with membrane proteins. Within the membrane, these interactions between lipids and proteins determine to a large extent the integrity of the membrane and the activity of the proteins. To study the properties of a membrane protein and the specific effects of lipid/protein and protein/protein interactions, it is essential to isolate the protein from the natural membrane and to reconstitute it into intact liposomes with a defined lipid composition (proteoliposomes). Studies employing these proteoliposomes have led to much of our understanding of the mechanisms and energetics of translocation processes. Amongst others, such studies have confirmed many of the predictions made by the chemiosmotic theory of Mitchell (Mitchell, 1961).

If a membrane protein is studied in detail, it is important that the protein is expressed at levels that are high enough to allow large-scale purification. The knowledge of protein expression in heterologous or homologous systems has increased tremendously during the last few years, and many membrane proteins have been amplified to levels that allow purification on a milligram scale from 1 liter of (bacterial) culture. This means that the level of expression should at least be 5% of total membrane protein. Published examples include: LacS, the galactoside transporter of *Streptococcus thermophilus* (Knol *et al.*, 1996); LmrP, the drug/H^+ antiporter (Putman *et al.*, 1999) and DtpT, the di and tripeptide transport protein of

Lactococcus lactis (Hagting et al., 1997); GalP, the galactose carrier (Spooner *et al.*, 1994); GusB, the glucuronide carrier (Liang *et al.*, submitted); MelB, the melibiose carrier (Pourcher *et al.*, 1995); NhaA, the Na^+/H^+ antiporter (Taglicht *et al.*, 1991); AraE, the arabinose carrier (Walmsley *et al.*, 1993); LacY, the lactose carrier (Viitanen *et al.*, 1986); $IICBA^{GlcNAc}$, the *N*-acetylglucosamine transporter (Mukhija and Erni, 1996); $IICB^{Glc}$, the glucose transporter (Buhr *et al.*, 1994), and IIC^{Man}, the mannose PTS (Mao *et al.*, 1995) of *Escherichia coli*. The amplified expression of several *E. coli* proteins is described in Ward *et al.*, 1999.

Recently, mutant strains of *E. coli* have been selected that appear to tolerate an increased expression of membrane proteins without, the commonly observed, toxic effect. (Miroux and Walker, 1996). From these studies, it is clear that over-expression is dependent on the genetic background of the expression host and that some factors, e.g., inefficient translation of mRNA, inhibit maximal expression in wild-type strains. Other important factors in over-expressing (membrane) proteins relate to conditions at which the cells are grown. Not only the concentration of the inducer (in case of an inducible system), but also other medium components, pH, osmolarity and temperature can have an marked effect on the expression of membrane proteins (Schertler, 1992), and therefore, expression needs to be optimized for every protein and expression host.

Homologous expression systems yield in general higher levels of protein and are more reproducible than heterologous systems. Furthermore, the amplified expression of membrane proteins form eukaryotic sources is often much more problematic then those of prokaryotic origin (for review, Grisshammer and Tate, 1995). We also stress that of the membrane proteins of prokaryotic origin, virtually nothing is known about those from archaeal source and relatively few systems for homologous expression in archaea are available.

An important development in membrane biology has been the purification of membrane proteins by affinity chromatography (Hochuli *et al.*, 1987; Crowe *et al.*, 1994). In most cases amino- or carboxyl-terminal histidine tags are engineered onto the proteins, but other affinity tags have been successfully employed as well. Even if expression levels are relatively low, these powerful methods allow the purification of proteins with very high specificity and efficiency, and proteins can in general be obtained with a purity of > 95% in a single step. The affinity purification techniques are relatively insensitive to the type of detergent and the presence of stabilizing additives (see below). (Affinity-)chromatography also offers a convenient way for the exchange of buffer and/or detergent (Casey and Teithmeier, 1993).

To accomplish selective extraction of (cytoplasmic) impurities and/or peripheral membrane proteins, one can treat crude membrane preparations with low concentrations of detergent. Selective extraction can be performed with ionic detergents like cholate and deoxycholate (Newman *et al.*, 1981; Knol *et al.*, 1996), but also with non-ionic detergents. For instance, one can obtain a more than 5-fold purification of a range of membrane proteins by treating membrane vesicles of *L. lactis* with low concentrations of $C_{10}E_8$ (~1%), prior to solubilization of the pelleted and pre-extracted membranes with DDM (Fang *et al.*, 1999).

Although proteoliposomes are widely used to study transport systems, little is known about the mechanism of (proteo)liposomes formation. This review deals with the detergent-mediated reconstitution of purified membrane proteins and the formation of bilayers from mixed micelles of lipid and detergent or lipid, detergent and protein. It is clear that there is no single reconstitution strategy that will work for every membrane protein, but accumulating information on protein/lipid/ detergent interactions will help in setting up and optimizing a reconstitution protocol for any given membrane protein. The understanding of the physical behavior of protein/detergent/lipid mixtures is also important for the field of membrane protein crystallization.

SOLUBILIZATION

INTRODUCTION

Solubilization is the first step in membrane protein purification. In general, membranes can be dissolved by amphiphiles such as fatty acid anions, bile salts, monoacylphospholipids, and synthetic detergents. By choosing the appropriate detergent and optimal solubilization conditions, it is in most cases possible to obtain membrane extracts in which the protein under investigation maintains its native structure. This implies that the detergent has to simulate the native environment of the membrane, and has to shield the hydrophobic parts from the aqueous phase without denaturing the protein (Helenius and Simons, 1975; Lichtenberg *et al.*, 1983; Lichtenberg, 1985).

PROPERTIES OF DETERGENTS

Detergent molecules can undergo reversible self-association and form micelles in aqueous solution. The hydrocarbon chain of the detergent molecules constitute the hydrophobic interior of the micelle which is separated from the polar environment by the hydrophilic headgroup. An important parameter of the detergents is the critical micelle concentration (CMC). The CMC is a measure of the free energy of stabilization of an amphiphile in associated form, and creates a barrier that must be overcome if dissociation from the aggregated state is to occur. Below the CMC a detergent is present in its monomeric form, whereas above the CMC aggregates are formed (micelles) which contain a more or less specific number of detergent molecules. The free energy of micellization can be described by two components (Tanford, 1974). The first component is an attractive force arising from the hydrophobic effect, which minimizes contact between the hydrocarbon chains and water. The second component consists of a repulsive force between the polar headgroups. The attractive force is increased by increasing the hydrocarbon chain length of the detergent, whereas the repulsive force can be decreased by using the appropriate counterions for the charged headgroups. The CMC decreases as a result of these changes.

The hydrocarbon chain length of the detergent is an important parameter in keeping the membrane protein in its native state. Another important property of detergents is the aggregation number, which is the average number of detergent molecules present in a micellar structure. The aggregation numbers of several detergents, including DDM, $C_{12}E_8$, and Triton X-100, are estimated to be much larger than those calculated for minimal spherical micelles on the basis of their alkyl chain length (Tanford, 1972). In fact, the hydrodynamic properties of these detergent micelles are most consistent with a disklike shape (Tanford *et al*., 1977; Robson *et al*., 1977). The aggregation number largely determines the size of the micelles, and this is particularly important if the protein is to be purified by size exclusion chromatography, that is, when the size of the protein/detergent complex has to differ significantly from that of free micelles.

Detergents commonly used can be classified on the basis of charge of the polar headgroup in neutral, ionic (cationic or anionic) or zwitterionic, and this charge can be of critical importance for the activity of the solubilized protein (Le Maire *et al.*, 1992; Banerjee *et al.*, 1995). The properties of the most commonly used detergents are summarized in Table 1.

(A) Ionic detergents. Ionic detergents like SDS (**anionic**) and cetyltrimethylammonium bromide (CTAB; **cationic**) are often efficient solubilizers, but they tend to denature the proteins.

(B) Neutral Detergents. (i) Polyoxyethylene detergents. Polyoxyethylene detergents are generally mild for the proteins, but they can be less efficient in their solubilization capacity. Detergents with short hydrocarbon chains, like C_8E_4, are more efficient in solubilizing membranes than those with longer hydrophobic moieties, but they often inactivate the proteins. Detergents with intermediate hydrocarbon chain length (C_{12}-C_{14}) appear most appropriate for retaining the proteins in their native state (Dean and Tanford, 1978; Lund *et al*., 1989; Casey and Reithmeier, 1993). Heterogeneity of the substances and/or

the presence of impurities can limit the use of polyoxyethylene detergents. It is often convenient that the detergent of choice does not absorb ultraviolet light in order to detect the protein at 280 nm and/or to perform spectroscopic studies. **(ii) Alkylglucosides.** In addition to octylglucoside, other alkyl glycosides, and in particular dodecylmaltoside (DDM), have successfully been used for the solubilization and reconstitution of numerous membrane proteins. **(iii) Lysolipids.** Because of their resemblance to membrane lipids, lysolipids represent another important class of detergents, but their use is limited because of a high price (Huang *et al.*, 1998).

(C) Zwitterionic detergents. Zwitterionic detergents form a heterogeneous group with N,N-dimethyldodecylamine oxide (DDAO; previously LDAO) and the sulfobetaines as the best-known examples (Hjelmeland, 1980).

(D) Steroid-based detergents. These compounds form a separate class, and their use quite often leads to less inactivation than when class B detergents with the same hydrophilic head group are used (Le Maire *et al.*, 1992). Steroid detergents, e.g., bile salts (cholate and deoxycholate) and CHAPS(O) have the advantage, as will be explained later, of a high critical micelle concentration (CMC).

Table I. Properties of Commonly Employed Detergents for Membrane Protein Reconstitution.

	Class[1]	M_r	Aggregation Number	Micellar Weight (kD)	CMC (mM)	R_{sat} (mol/mol)	R_{sol} (mol/mol)	Data From[2]
SDS	A	288	62-101	18-29	8.2	-	-	1
CTAB	A	365	169	62	0.92	-	-	2
Triton X-100	B*i*	625	73-140	46-90	0.21	0.64	2.5	2,3
C10E6	B*i*	423	32	14	0.9	-	-	3
C10E8	B*i*	511	-	-	0.28	-	-	4
C12E6	B*i*	451	105	50	0.056	-	-	4
C12E8	B*i*	539	90-120	49-65	0.087	0.7	2.2	3,4
Tween 80	B*i*	1300	60	76	0.012	-	-	1
DDM	B*ii*	511	98-140	50-71	0.16	1.0	1.6	1,5
DM	B*ii*	483	-	-	1.4	-	-	4
Octylglucoside	B*ii*	292	27	8	25	1.3	2.6	5
Octylthioglucoside	B*ii*	308	-	-	9	2.8	5.8	6
Lysolecithin	B*iii*	-	-	-	0.2	-	-	7
DDAO	C	229	76	17	0.48	-	-	1
Cholic acid	D	408	5	2	3	0.3	0.9	1
Deoxycholic acid	D	392	55	23	0.91	-	-	1
CHAPS	D	615	10	6	8	0.4	1.04	1,8
CHAPSO	D	631	11	7	8	0.38	0.73	1,8

[1] *See text;*

[2] *References: [1] Helenius et al., 1979; [2] Helenius and Simons, 1975; [3] Le Maire et al., 1992; [4] Casey and Reithmeier, 1993; [5] Rosevaer et al., 1980; [6] Saito and Tsuchiya, 1984; [7] Lasic, 1993; [8] Cladera et al., 1997. Solution properties of detergents depend strongly on temperature, pH, counterion, and ionic strength. The data also depend on the particular methods used to determine the CMC (ANS-fluorescence or surface tension measurements) or the micellar size (gelfiltration, analytical ultracentrifugation or light scattering).*

THE PROCESS OF MEMBRANE SOLUBILIZATION

Helenius and Simons proposed that solubilization of membranes by detergents can be subdivided in three distinct phases. In the first phase, the detergent binds to the membranes, which is followed by partitioning in the lipid environment. In the second phase, the lamellar structures are disrupted and mixed micelles of lipid, protein and detergent co-exist with bilayer structures. In the third phase, that is with increasing detergent concentration, the bilayer structures are completely disrupted and proteins and lipids are only present in micelles (Helenius and Simons, 1975). The three stage model of membrane solubilization was confirmed by Lichtenberg who monitored the effects of increasing detergent concentrations on the turbidity of a liposome suspension (Fig. 1) (Lichtenberg, 1985; Rigaud *et al.*, 1995; Knol *et al.*, 1996; De La Maza and Parra, 1997).

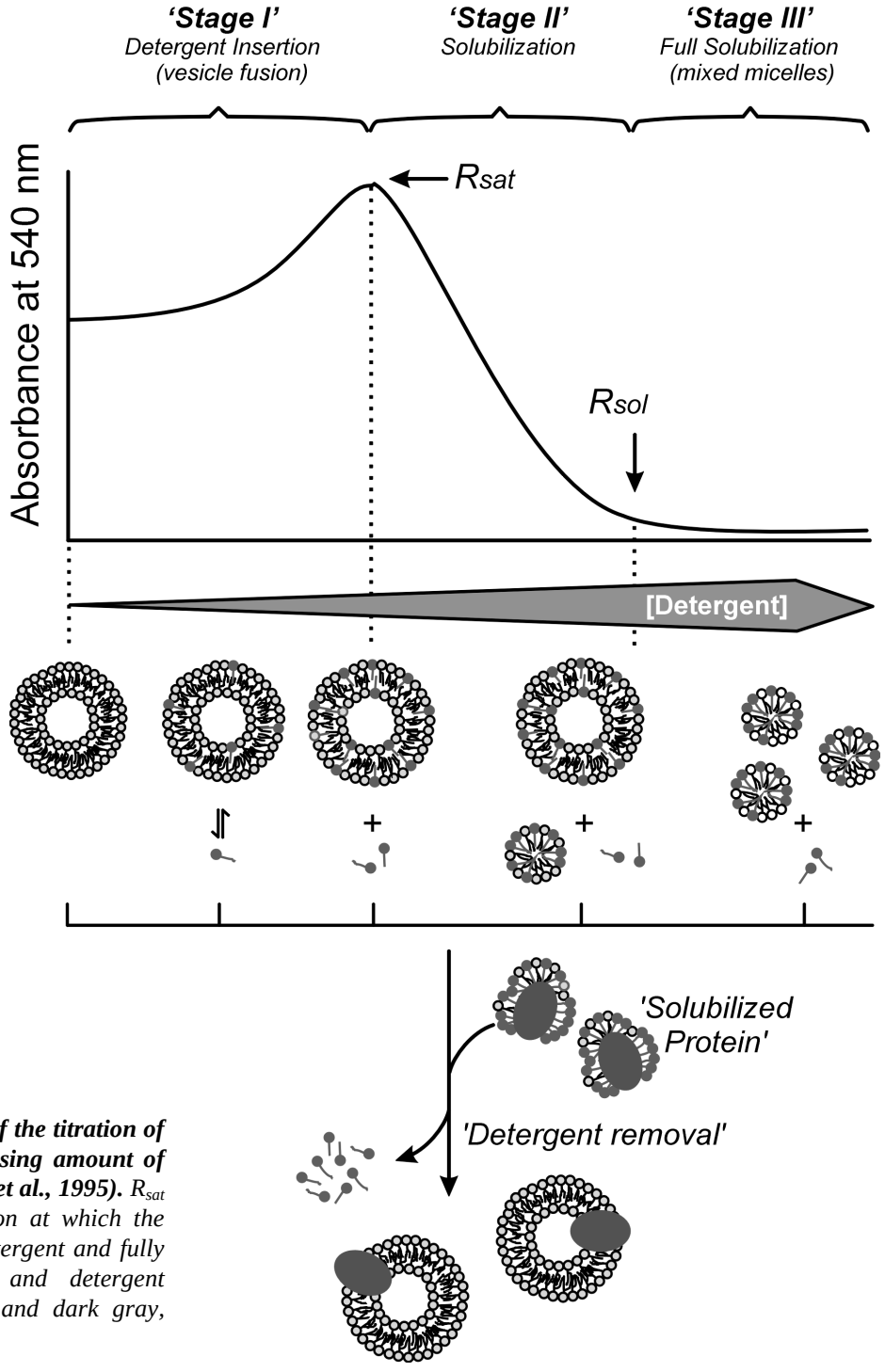


Figure 1. Schematic illustration of the titration of preformed liposomes with increasing amount of detergent (modified after Rigaud et al., 1995). *R_{sat} and R_{sol} refer to the concentration at which the membranes are saturated with detergent and fully solubilized, respectively. Lipid and detergent molecules are depicted in light and dark gray, respectively.*

Detergent incorporation into the lipid bilayer (Phase I), results in a swelling of the liposomes which can be observed as an increase in OD_{540}. Detergent partitioning precedes cooperative binding and solubilization, and at the transition to cooperative binding, detergents like $C_{12}E_8$, Triton X-100 and DDAO, induce fusion of small liposomes which results in the formation of larger liposomes. This membrane fusion already occurs at detergent concentrations below R_{sat} and is observed as a strong increase in OD_{540} (Lasch, 1995; Kragh-Hansen *et al*, 1998). The increase in OD_{540} that is observed with DDM is explained by dramatic changes in liposomal structure rather than liposome fusion; this aspect will be discussed later.

The free detergent concentration in solution reaches the CMC at R_{sat}, and the critical effective detergent to lipid molar ratio (R_e) at which the liposomes start to dissolve depends on the CMC and bilayer/medium distribution coefficients of the detergent. At higher detergent concentrations the liposomes disintegrate (Phase II), and a transition takes place from bilayer structures to mixed micelles. Finally, at R_{sol}, the liposomes are completely solubilized (Phase III), and further increases in detergent concentration only affect the ratio of detergent and lipid molecules present in the micelles (Lichtenberg, 1985). Turbidity measurements have been used to monitor the process of membrane solubilization, and plots of optical density versus detergent concentration provide direct information about R_{sol} and R_{sat} (Table I). The information obtained from the solubilization is of immediate importance for an understanding of the reconstitution process as will be discussed below in more detail.

INTERACTIONS BETWEEN DETERGENTS AND PROTEINS

Unlike to what is often assumed, the amount of detergent that binds to a membrane protein is not similar to the number of molecules present in the free micelle. The number of detergent molecules bound to a protein depends on the shape and size of the hydrophobic belt of the protein, that is the portion of the molecule that is normally in contact with the lipids. It is also thought that the detergent molecules around a protein are not arranged in a micellar but rather lamellar manner (Timmins *et al.*, 1988; Møller and le Maire, 1993). Finally, even when the detergent concentration is lowered to far below the CMC, detergent molecules bound to a hydrophobic protein can be retained for long periods of time as was demonstrated for the binding of $C_{12}E_8$ and Triton X-100 to Ca^{2+}-ATPase (Le Maire *et al.*, 1983; Lund *et al.*, 1989). It thus appears that the affinity of proteins for detergents can be very high, which has consequences for the process of membrane reconstitution.

Once the protein has been solubilized and is to be studied in its solubilized state, it is crucial that it retains its native conformation. The choice of the detergent for solubilization and reconstitution often depends on the stability of the protein in the specific detergent and buffer components. In some cases, however, the integrity of the protein can be improved by adding stabilizing agents such as phospholipids, compatible solutes and/or specific ligands (Newman and Wilson, 1980; Newman *et al.*, 1981; Chen and Wilson, 1984; Cornell and MacLennan, 1985; Maloney *et al.*, 1992; Poolman and Konings, 1993; Tanio *et al.*, 1998). For example, when the glucose transporter from erythrocyte membranes (Glut1) is solubilized with octylglucoside, the addition of PC is essential for maintaining activity, whereas the protein is stable in $C_{12}E_8$ without the addition of PL (Haneskog, 1996). Cholinephosphotransferase from rabbit skeletal muscle solubilized with cholate, deoxycholate, and octylglucoside, is rapidly inactivated, unless specific additives are present in combination with the detergent. In this case, the diacylglycerol and glycerol were found to be effective stabilizers. High concentrations (10-20%) of glycerol and higher polyols (erythritol, xylitol, sorbitol), sugars (glucose, trehalose), and certain amino acids (glycine, proline, betaine) generally protect (membrane) proteins against denaturation (Yancey *et al.*, 1982; Maloney and Ambudkar, 1989). It should be stressed, however, that glycerol may have adverse effects when detergents with polyoxyethylene headgroups are used (Friesen and Poolman, manuscript in preparation)

RECONSTITUTION

INTRODUCTION

To study the catalytic activity of a purified membrane protein, it is in many cases necessary to reconstitute the protein in phospholipids. The use of proteoliposomes has numerous advantageous over less well-defined membrane systems. Competing reactions present in cell or membrane vesicle systems are eliminated, and one obtains a clear control over the enzymatic parameters to be determined. The effect of specific lipids on the activity of the protein can be studied, interactions with other proteins can be circumvented, and by imposing (artificial) ion gradients the energetics can be controlled and measured accurately. It is also possible to co-reconstitute membrane proteins, for instance, the combination of energy-generating and -consuming systems, e.g., cytochrome *c* oxidase or the light driven proton pump bacteriorhodopsin together with a membrane transport protein allows one to study the interdependence of the systems in terms of energetics (Rigaud *et al.*, 1985; Richard *et al.*, 1995).

Important parameters for the choice of lipids include the phase transition temperature of the lipid, the ability of the lipid to form lamellar structures (bilayer), the integrity and permeability of the bilayer formed, and the hydrophobic thickness of the bilayer (In't Veld *et al.*, 1993). Sometimes specific lipids are essential to obtain maximal activity, for example, PE or PS in case of the lactose permease of *Escherichia coli*, and cardiolipin in case of cytochrome oxidase (Seto-Young *et al.*, 1985; Bogdanov and Dowhan, 1995; Paradies *et al.*, 1997).

CHOICE OF LIPID AND INTEGRITY OF MEMBRANES

In membranes with a weight ratio of phospholipids to protein of 1 and an average protein molecular weight of 50,000 the molar lipid/protein ratio is about 60. Considering the sizes of the molecules, it can be estimated that at any time about 50% of the lipids are adjacent to a protein molecule (Gennis, 1989). These are the so-called annular lipids. The remaining 50% of the lipids form the membrane with its typical bilayer properties.

Lipids have two important functions for integral membrane proteins. Firstly, they provide an environment in which the membrane proteins resides, and secondly lipids can act as allosteric effectors of enzyme activity. Thus, not only general membrane properties such as fluidity and permeability are important for the functioning of a protein, but also specific protein-lipid interactions (de Kruijff, 1997). The variation in head groups, chain lengths and degrees of chain unsaturation results in the existence of thousands of different biological lipids, of which several hundreds can be present in the same membrane (Devaux and Seigneuret, 1985). The enormous diversity of lipids can be rationalized when one assumes that membrane protein function is modulated by the local fluidity of the bilayer matrix and/or the detailed composition of the annular lipids at the protein-lipid interface. Indeed, most integral membrane proteins require a fluid (liquid-crystalline) phospholipid environment for function (Warren *et al.*, 1975). In spite of intensive efforts, there is surprisingly little evidence that the activity of any integral membrane protein is totally dependent on one specific lipid (Cullis and De Kruijff, 1979; de Kruijff, 1997; Epand, 1998). Some well-documented examples of critical effects of specific lipids on enzyme activity are summarized:

- PE or PS is required for proton motive force-driven lactose uptake by the lactose carrier, LacY, of *E. coli* (Seto-Young *et al.*, 1985; Chen and Wilson, 1987).
- Aminophospholipids are required for maximal activity of the branched amino acid carrier of *Lactococcus lactis* (In't Veld *et al.*, 1991; 1993).
- Cardiolipin is essential for maximal cytochrome *c* oxidase activity (Bogdanov and Dowhan, 1995; Paradies *et al.*, 1997).

- Specific native lipids are essential for optimal activity and trimer formation of bacteriorhodopsin (BR) from *Halobacterium salinarium.* Brief treatment of purple membrane with dilute detergent solutions causes a major disruption of the BR photocycle without disrupting the trimer structure of BR. Full restoration of activity is obtained when squalene (SQ) plus phosphatidyl glycerophosphate (PGP) are added (Joshi *et al.*, 1998; Sternberg *et al.*, 1992; Tanio *et al.*, 1998).
- PE or PG, but not PC activate the leucine carrier protein of *Pseudomonas aeruginosa* (Uratani, 1985; Uratani and Aiyama, 1986).
- PC with certain tail lengths and degree of unsaturation activate hydroxybutyrate dehydrogenase from the inner mitochondrial membrane (Grover *et al.*, 1975).
- Negatively charged phospholipids are required for light-driven ATP-synthesis by reconstituted bacteriorhodopsin and H^+-transporting ATP-synthase from thermophilic *Bacillus PS3* (TF_0F_1). Cholesterol induces a fourfold increase in ATP-synthase activity with a concomitant 65% decrease in the K_m for ADP, suggesting that sterols can modulate catalytic events (Pitard *et al*., 1996).
- The pre-synaptic sodium-dependent serotonin transporter requires cholesterol for maximal binding activity (C.G. Tate, pers. comm.).

The hydrocarbon length (C_{16}-C_{20}) and degree of unsaturation of the acyl chain are more important for the activity of sarcoplasmic reticulum Ca^{2+}-ATPase than the composition of the head groups (Pikula *et al.*, 1994). In case of the leucine carriers of *Lactococcus lactis* and *P. aeruginosa*, lipids with an acyl chain length of about 18 are optimal, also indicating an important role for the membrane thickness in transport activity (In't Veld *et al.*, 1991; Uratani *et al.*, 1987). If the transmembrane spanning segments of a protein are not correctly embedded in the bilayer, because of an inappropriate bilayer thickness, one could expect conformational changes in the protein that affect the activity.

The structure of LacY of *E. coli* has been studied by Fourier transform infrared spectroscopy and the average tilt angle for the helices in the proteoliposomes was 33^o under optimal conditions (molar lipid to protein ratios of 800 or higher) . In this proteoliposomal system with lipid to protein ratios that are still much higher than in the native membrane, the structure and activity of LacY strongly depended on the concentration of the protein in the membrane (Le Coultre *et al.*, 1997). With decreasing lipid to protein ratios, the activity decreased in a manner that correlated with a decrease in the lipid order parameter and an increase in the average helical tilt angle (51^o). How an increased protein concentration affects the order and integrity of the lipid bilayer is unknown, but a strong dependence of transport activity on the concentration of the protein in the membrane has been observed for other systems as well (Knol *et al*., 1999; Fang *et al*., 1999).

RECONSTITUTION WITHOUT (AN EXCESS OF) DETERGENT

Several strategies for the reinsertion of membrane proteins into liposomes have been described that do not require the use detergents. These methods have been reviewed extensively (Jain and Zakim, 1987; Rigaud *et al.,* 1995) and they will only be summarized here. Firstly, proteins, from which most of the detergent has been removed, can sometimes be incorporated directly into preformed liposomes of defined composition by mechanical means, that is, sonication (Racker, 1979), passage through a french press (Barenholzt *et al*, 1979) or freeze/thawing (Kasahara and Hinkle, 1977). Alternatively, one may use liposomes of very small size and trigger the insertion by amphipathic molecules such as cholesterol, short chain PC or fatty acids. The presence of cholesterol in small unilamellar liposomes of dimyristoylphosphatidylcholine (DMPC) catalyzes the insertion of several integral membrane proteins. Bacteriorhodopsin from *Halobacterium salinarium*, UDPglucuronosyltransferase from pig liver microsomes, and cytochrome *c* oxidase from beef heart mitochondria can be inserted into liposomes containing cholesterol. It appears that cholesterol lowers the energy barrier for the insertion of integral

membrane proteins into these bilayers (Scotto and Zakim, 1986; Eytan *et al.*, 1976; Jain and Zakim, 1987). Although some successes have been reported, these procedures are not generally applicable and often lead to protein aggregation (Kagawa and Racker, 1971; Racker, 1979; Eytan, 1982).

Protocols for the preparation of liposomes with the use of organic solvents have also been used to reconstitute membrane proteins. In this case large proteoliposomes are formed in water when the excess of organic solvent (e.g., pentane, hexane or diethyl ether) is removed from an emulsion of lipid/protein and aqueous buffer. Methods used include: ethanol injection (Batzri and Korn, 1973), ether infusion (Deamer and Bangham, 1976) and reverse-phase evaporation (Szoka and Paphadjopoulos, 1978). The only examples of successful reconstitution by organic solvent-mediated reverse-phase evaporation are bovine rhodopsin and bacteriorhodopsin (Darszon *et al.*, 1979; Rigaud *et al.*, 1983). In many cases, however, the protein is denatured by the organic solvents before it is inserted into the lipid bilayer.

In conclusion, methods based on reconstitution without (an excess of) detergent yield, besides a low efficiency, proteoliposomes with a heterogeneous size distribution, a large proportion of multilamellar structures and a heterogeneous protein distribution over the proteoliposomes. It is therefore not surprising that in the fast majority of papers about the reconstitution of membrane proteins specific detergents are used.

DETERGENT-MEDIATED RECONSTITUTION

CLASSICAL METHODS

The most frequently applied method for membrane protein reconstitution involves the co-solubilization of membrane protein and phospholipids, after which the detergent is removed, leading to spontaneous formation of liposomes with bilayer membranes in which protein is incorporated. Often octylglucoside is used as solubilizing agent, as removal of this detergent is easily achieved by dilution or dialysis. Octylglucoside, but also cholate, deoxycholate, CHAPS and CHAPSO, have the advantage that these detergents have a high CMC, and their small micellar sizes allow one to perform reconstitutions via dialysis, dilution or gel filtration (Baron and Thompson, 1975; Shanahan and Czech, 1977; Racker, 1979; Mimms *et al.*, 1981; Henderson, 1983; Schurtenberger *et al.*, 1984; Viitanen *et al.*, 1986; Ruan *et al.* 1992; Cladera *et al.*, 1997). Detergents can also be removed by adsorption onto hydrophobic resins (SM2 Biobeads or Amberlite XAD), or by binding to cyclodextrins (Degrip *et al.*, 1998). Especially for detergents with a low CMC and high aggregation numbers, e.g., Triton X-100, DDM and polyoxyethylene detergents, which are not readily removed by gel-filtration or dialysis, the development of absorption resins has great advantages in reconstitution experiments (Holloway, 1973; Allen *et al.*, 1980; Ueno *et al.*, 1984).

The size distribution of the proteoliposomes formed depends on the fusogenic properties of the detergent and the ratio of detergent to phospholipid, but even more on the method and rate of detergent removal. If detergent removal is fast (e.g., dilution), the detergent-saturated liposomes have no time to fuse and will form small structures. On the other hand, when detergent removal is slow (e.g., dialysis), the detergent-saturated liposomes are long-lived and fusion leads to large proteoliposomes. The rate of detergent removal also affects the lamellarity of the (proteo-)liposomes. A large fraction is multilamellar when the removal is slow, whereas a large proportion is homogeneous and unilamellar in case of rapid detergent removal (Lambert *et al.*, 1998). Since detergent dialysis takes much longer than detergent dilution, it may also lead to more protein denaturation or aggregation (Levy *et al.*, 1992; Lasch, 1995). Factors that play a role in the fusion process are (i) ion concentrations (like Ca^{2+} and Mg^{2+}), (ii) type of phospholipid, (iii) pH, and (iv) temperature (Oku and MacDonald, 1983; Siegel, 1993).

FORMATION OF MICELLES

In order to understand the process of membrane reconstitution from a mixture of detergent, lipid and protein, it is important to know what the driving forces and constraints of micelle and bilayer formation are. These parameters have been reviewed by Gennis (Gennis, 1989), and only the most important factors will be summarized here.

The favorable interactions between the polar headgroups of amphiphilic molecules, like detergents, and water leads to the formation of globular aggregates. When the solubility for the monomeric form is reached, it will form aggregates (micelle formation) dispersed throughout the solution. Mixed micelles of lipid and detergent can have a range of sizes and shapes, like spherical, globular or rodlike. Phospholipids can also form bilayers, which is essentially another micellar form. Biological phospholipids have two long alkyl chains per molecule that strongly favor a bilayer state; the CMC for such lipids is $<10^{-10}$ M, which is several orders of magnitude lower than that of detergents (Table I).

The geometry of an amphipathic molecule plays a critical role in the molecular packing of the amphipathic monomers (**Fig. 2)**. At the CMC, single chain surfactants such as detergents aggregate into micelles. The structure of micelles can be described by a dimensionless packing parameter (P), which is related to the shape of the detergent monomer:

$$P = V / (A_o l_c)$$

where V is the volume of the hydrocarbon chain, A_o is the optimal surface area per headgroup in the aggregate, and l_c is the length of the alkyl chain (Israelachvili *et al.*, 1976). A_o is dependent on the solution conditions, especially ionic strength in the case of charged molecules, whereas l_c determines the upper limit of the micellar size such as the radius of a spherical micelle or the thickness of a bilayer in case of double-chain lipids.

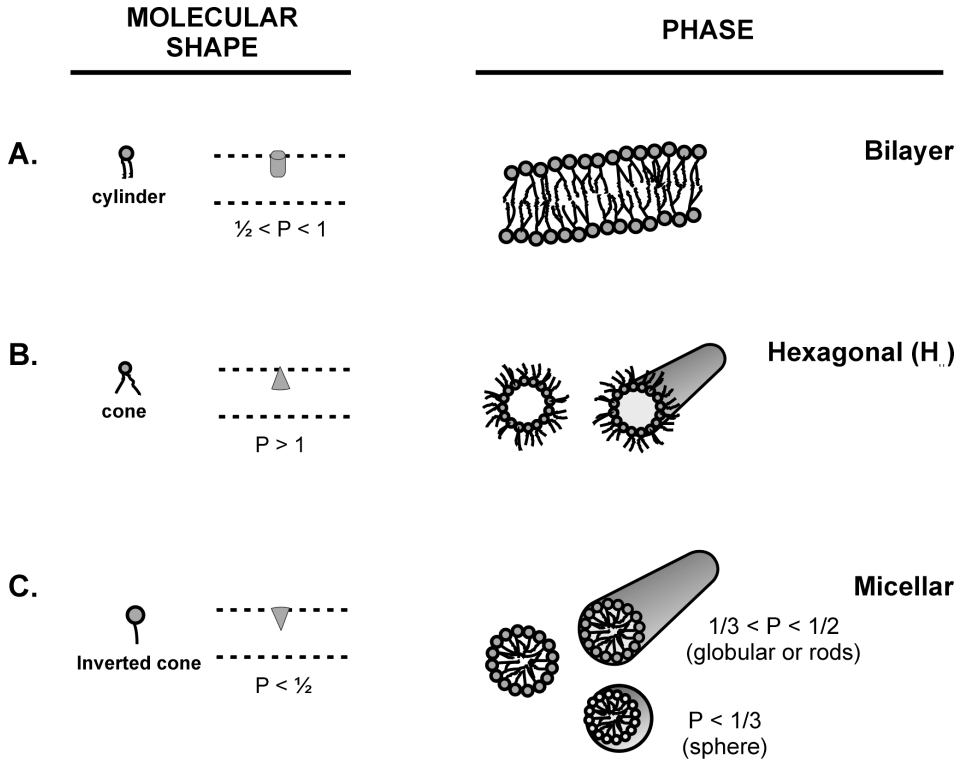


Figure 2. Molecular shape of amphipathic molecules and macro-molecular structures formed upon aggregation. *(**A).** Lipids like PC, PS, PI, PG, CL and sphingomyelin have a cylindrical molecular shape and bilayer structures (lamellar or L_α-phase) form upon aggregation. (**B).** Lipids like unsaturated PE, PS (pH<4), and Ca^{2+}-CL have a cone-like shape and these molecules form inverted structures. The relative small polar headgroups are facing inwards and the cylinders will pack into hexagonal arrays (inverted or H_{II}-hexagonal). (**C).** Detergents with short hydrophobic tails and large headgroups (inverted cone) will form spherical micelles with the polar groups facing outside (micellar). When the packing parameter is between 1/3 and 1/2, the monomer is wedge-shaped, and the molecules form thread-like micelles (rods).*

For single-chain surfactants, the polar head is relatively large with respect to the non-polar part, and these molecules pack into spherical or rodlike micelles. Many double-chain lipids have a P of about one and these molecules pack best into lamellar structures, whereas lipids with relatively small polar heads (P > 1, e.g. PE) tend to form inverse structures (Fig. 2).

FORMATION OF BILAYERS

The formation of bilayers upon removal of detergent is driven by three thermodynamic forces. The major stabilizing factor is the hydrophobic force, which results in minimal contact between the nonpolar regions of the lipids and water. Exclusion of water molecules from the hydrophobic tails leads to an increase in entropy, which is also called the hydrophobic effect (Tanford, 1973). Secondly, the van der Waals forces form short, weak attractive interactions between hydrocarbon chains via polarizable electrons (induced dipoles). Finally, hydrogen bonding between headgroups also contributes to the stability of the bilayer, and in case of anionic lipids the intermolecular bridging by divalent cations is important (Boggs, 1983). Biological membranes are usually in the liquid-crystalline phase, but also non-lamellar phases can be found depending on factors like temperature, fatty acid composition, pH or the presence of divalent cations. Non-lamellar-forming lipids can significantly affect the physical properties of membranes and thereby affect the activity of membrane proteins (Epand, 1998).

In the lamellar gel phase (L_β), which is formed at temperatures below the gel to liquid-crystalline phase transition temperature, the fatty acids are packed tightly and the chains are maximally extended. The bilayer thickness is greater than in the liquid-crystalline phase and also the density is slightly higher. In biological membranes, however, the bulk of lipids is present in the liquid-crystalline phase (L_α) and is characterized by an ordered two-dimensional organization with considerable disordered arrangement of the acyl chains. When lipids are in the hexagonal phase, H_I or H_{II}, the lipids are organized in cylinders with the polar head groups at the outside or the inside, respectively. The cylinders are packed in a hexagonal pattern. An important parameter for the phases of membranes is the temperature at which the transition between the gel to liquid-crystalline phase takes place (T_c). Below T_c the lipids are packed in the lamellar gel phase (L_β), whereas above T_c they are more disordered and fluid (L_α) (de Kruijff, 1997; Lasic, 1998).

It is difficult to predict what structures will be formed in lipid/detergent mixtures, and in order to understand bilayer formation and membrane protein reconstitution, it is important to study the macromolecular structures formed by lipid/detergent/protein mixtures during solubilization and reconstitution.

RECONSTITUTION INTO PREFORMED LIPOSOMES: THE REVERSE OF SOLUBILIZATION

PREPARATION OF LIPOSOMES

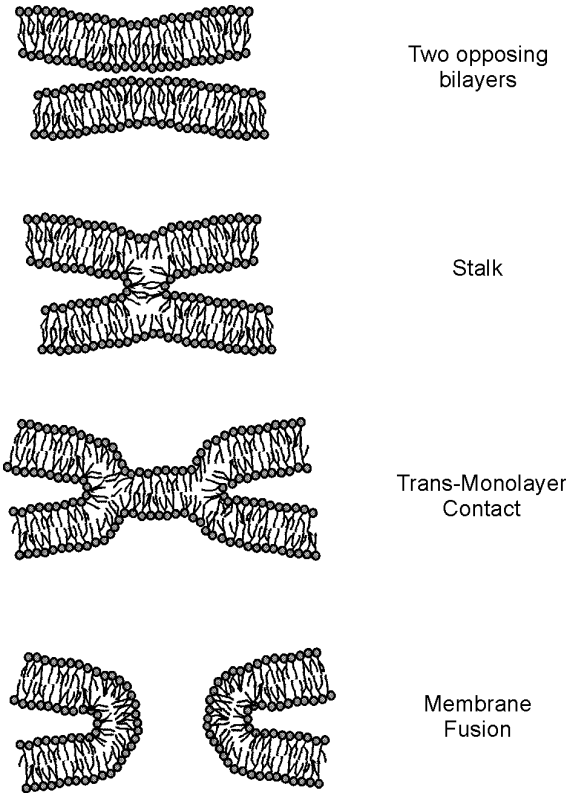
Phospholipids are not readily dissolved in water and when these amphiphiles are mixed with water most of the lipids are not fully hydrated. Hydration is facilitated when the surface-to-volume ratio is increased, which can be done by preparing a thin lipid film by evaporation from an organic phase or by using a fine powder of spray-dried lipid from an organic solvent. The hydration of thin lipid films results in the formation of heterogeneous dispersions of liposomes with predominantly large multilamellar vesicles (MLV). In case of charged phospholipids the fraction of smaller and unilamellar structures is larger due to electrostatic repulsion which, in general, improves the solubility. In order to produce homogeneous large unilamellar liposomes (LUV) or small unilamellar liposomes (SUV) from MLV, some energy (mechanical, electrostatical or chemical) must be dissipated into the system. Mechanical means of converting MLV into LUV or SUV are often preferred and include, extrusion of the MLV through filters

with pores of defined diameters, the use of the “French press”, or sonication of MLV (Olson *et al.*, 1979; Mayer *et al.*, 1986; MacDonald *et al.*, 1991; Barenholzt *et al.*, 1979; Racker, 1979). Electrostatic treatments of MLV include, changes of counter ions, pH, or ionic strength. Also short chain diacyl lipids, some lysolipids or detergents can induce the transformation from multi-lamellar to uni-lamellar liposomes chemically, because of their fusogenic properties (Lasic, 1993).

For the preparation of liposomes it is also possible to transfer lipids from the organic phase directly into water, upon which the lipid molecules will aggregate to accommodate to the new solubility conditions. This can for example be done by emulsification, injection, solvent dialysis, or extraction. In addition, lipids can also be dissolved into an aqueous solution with the aid of co-solubilizers, such as detergents, amphiphilic solvents, or chaotropic ions. Upon the removal of the co-solubilizer normally unilamellar liposomes are formed (Lasic, 1993).

Liposomes are not colloidally stable and, dependent on their composition, they slowly aggregate and fuse into large and more lamellar structures (Lasic, 1998). Although an attractive van der Waals force between two phospholipid bilayers exists, the strong repulsive electrostatic interactions between liposomes with negatively charged phospholipids prevent unilamellar phospholipid liposomes to fuse rapidly. Increasing the size of the (proteo)liposomes by fusion allows the generation of larger structures, which is often important for further functional analysis of the reconstituted membrane protein (Lasic, 1988; Kasahara and Hinkle, 1977; Driessen and Konings, 1993). Liposome fusion can be enhanced by repeated cycles of freeze-thawing. In the process of rapid freezing, water molecules crystallize on the charged phospholipid interface, forming two frozen planes separated by the hydrophobic core of the membrane. The bilayer is locally disrupted and the exposed hydrophobic cores fuse to form large liposomes during slow thawing (Pick, 1981). Dehydration of liposomes also facilitates the fusion process. Phospholipids bind water, and the oriented layer of water molecules at the surface of the bilayer must be removed to allow the two bilayers to come into direct contact. Dehydration is energetically unfavorable, but some agents like Ca^{2+}, polyethylene glycol, detergents (see below) and also specific peptides or proteins can catalyze fusion by dehydration of the lipid bilayer. Phospholipids such as PE are less hydrated and, consequently, liposomes containing this lipid fuse more readily than those without PE (Kachar *et al.*, 1986).

Figure 3. Schematic representation of the structures formed during the fusion of bilayers.



The stalk theory (Siegel, 1993) predicts that intermediate structures of membrane fusion are the same as those involved in the transition from the lamellar (L_α) to the inverted (H_{II}) phase (Fig. 3), and these predictions are supported by time-resolved cryo-electron microscopy studies (Siegel and Epand, 1997). The structures with the lowest energy formed between membranes are called stalks. These stalks can lead to transmonolayer contacts (TMC’s) and these intermediate structures are critical in the fusion process, because they can transform into so-called fusion pores leading to the fusion of two bilayers (Siegel, 1999). The stability of the intermediate TMC

structure is important for the rate of membrane fusion, and depends on the composition of the bilayer and the properties of the specific lipid (or protein and detergent) molecules (Siegel, 1999). Inverted cone-like lipids like PE favour these structures, whereas DDM has a destabilizing effect on the TMC. This could explain the lower rate of fusion between liposomes, when they are titrated with DDM. This contrasts the situation with Triton X-100, a cylindrical molecule that aids liposome fusion, possibly through the stabilization of the TMC.

INSERTION OF PROTEINS INTO PREFORMED LIPOSOMES

In order to gain a better control of the formation of proteoliposomes, methods have been developed in which membrane proteins are inserted into preformed liposomes that have been destabilized with detergent. The method builds on the notion that reconstitution is the reverse process of membrane solubilization (Rigaud *et al.*, 1988; Levy *et al*., 1990a; Levy *et al*., 1990b). Unilamellar liposomes are slowly titrated with increasing concentrations of detergent, and the physical state of the liposomes is followed by measuring the turbidity of the suspension. Membrane reconstitution of an integral membrane protein can often be optimized by inserting the solubilized protein into liposomes destabilized by detergent concentrations in the range of R_{sat} to R_{sol} (Fig.1). When detergent is subsequently removed from phospholipid-detergent mixed micelles by adsorption to polystyrene, the apparent particle size and turbidity pass through a maximum similar to what is observed during the stepwise solubilization of liposomes (Ueno, 1989). During removal of octylglucoside or $C_{12}E_8$, liposome formation took place in three distinct phases with striking similarities between the critical detergent to lipid ratios at which phase transformations during solubilization and reconstitution occurred (Levy *et al.*, 1990B). Similar conclusions have been drawn for Triton X-100 mediated reconstitutions (Rigaud *et al*., 1995).

A number of advantages can be recognized of reconstituting membrane proteins into detergent-destabilized liposomes over membrane reconstitution from soluble mixtures of detergent, lipid and protein: (i) one has a better control over the incorporation of protein into lipid bilayers; (ii) it is easier to achieve membrane reconstitution with a uniform direction of the protein (see below); (iii) the protein is faced with lower detergent concentrations for a shorter period of time; (iv) the losses of protein (aggregation) and lipids are generally low.

The optimal conditions for reconstitution into preformed liposomes have been studied in detail for bacteriorhodopsin from *Halobacterium salinarium* and the H^+-transporting ATP synthase from thermophilic *Bacillus PS3* (TF_0F_1). Liposomes prepared from PC plus PA were titrated with various amounts of Triton X-100, octylglucoside, $C_{12}E_8$, sodium cholate or sodium deoxycholate, and the incorporation of both proteins into the liposomes mediated by the detergents was studied. After removal of detergent by means of SM-2 Bio-Beads, the light-driven ATP synthase activities of the resulting proteoliposomes were analyzed, and the most efficient co-reconstitutions were obtained when octylglucoside or Triton X-100 were used to insert the proteins into detergent-saturated (R_{sat}) liposomes. The optimal conditions for the incorporation into liposomes of bacteriorhodopsin from *H. salinarium* and the H^+-ATP synthase from spinach chloroplasts, were realized when CHAPS or CHAPSO were used at concentrations representing R_{sol}, that is with mixed micelles of detergent and lipids (Rigaud *et al.*, 1988; Richard *et al.*, 1990; Cladera *et al.*, 1997).

Such systematic studies have also successfully been performed for a number of other membrane proteins, that is the Ca^{2+}-ATPase from sarcoplasmic reticulum (Levy *et al.*, 1992), the lactose transport protein, LacS, from *S. thermophilus* (Knol *et al.*, 1996), the glutamate carrier GltT from *Bacillus stearothermophilus* (Gaillard *et al.*, 1996), the erythrocyte membrane anion exchanger, band 3 (Boulter *et al.*, 1996), photosystem I reaction center from cyanobacterium *Synechocystis sp.* (Cladera *et al.*, 1996), the di- and tripeptide transport protein DtpT from *Lactococcus lactis* (Hagting *et al.*, 1997), the peripheral

membrane protein glycosyl-phosphatidylinositol-alkaline phosphatase from bovine intestine (Angrand *et al.*, 1997), and the proline carrier protein, PutP, from *E. coli* (Jung *et al.*, 1998).

Although the number of systematic reconstitution trials is still limited, it is possible to discern some generalizations from these studies. Firstly, membrane protein reconstitution mediated by octylglucoside is often optimal at R_{sat}, whereas the lipid detergent ratio seems to be less critical if the reconstitution is mediated by Triton X-100. Steroid based compounds, on the other hand, require full solubilization (R_{sol}) of the liposomes in order to be maximally efficient in reconstitution (Rigaud *et al.*, 1995). This suggests that there are different mechanisms by which detergents mediate the formation of proteoliposomes upon the removal of detergent.

MOLECULAR MECHANISMS OF SOLUBILIZATION AND RECONSTITUTION

The absorbance (turbidity) measurements indicate the stage of liposomes solubilization by detergents, but they do not give information about the mechanism of solubilization or the macromolecular structures formed. Indeed by employing cryo-TEM and other methods, it has been shown that various non-ionic detergents affect the liposome structures in a completely different manner (Knol *et al.*, 1998; Lambert *et al.*, 1998). The observed differences are explained in more detail for Triton X-100 and DDM, but the results presented are also relevant for other detergents. It is important to keep these differences in mind when a detergent and optimal conditions are chosen for the reconstitution of a particular membrane protein.

TRITON X-100

When Triton X-100 is added to a liposome suspension, the mixture equilibrates rapidly and upon further addition of detergent the structures shift from a liposomal towards a micellar state. Triton X-100, but also $C_{12}E_8$, have a high degree of lipophilicity, that is, these detergents are easily dissolved in a variety of organic solvents, and this might explain the rapid equilibration of the detergent in the bilayer. This is in agreement with the observation that the flip-flop of $C_{12}E_8$ and Triton X-100 from the outer to the inner leaflet of the membrane occurs within fractions of a second (Le Maire *et al.*, 1987; Kragh-Hansen *et al.*, 1998).

The effect of Triton X-100 on the liposome to micelle transition has been studied by cryo-TEM, freeze-fracture-EM and dynamic light scattering, and it appears that liposomes are solubilized by the progressive formation of mixed micelles within the bilayer without causing complex intermediate aggregates. Surprisingly, small liposomes, composed of egg-PC or *E. coli* lipids plus 25% PC, are still found at R_e values that are close to R_{sol} (Phase II of the solubilization process; Fig.1). It seems that lipids are extracted from the bilayers by diffusion of mixed micelles of lipid and detergent from the bilayer without disrupting the liposomes (Knol *et al.*, 1998). The decrease in OD_{540} with increasing Triton X-100 concentrations parallels a decrease in the diameter of the liposomes, but the bilayer structures remain intact as judged by cryo-TEM. The shape of the mixed micelle that is liberated from the bilayer is elongated and becomes spherical when the detergent proportion in the system increases (Lopez *et al.*, 1998).

It has been speculated that Triton X-100 has a molecular shape that allows bilayers to exist at saturating detergent concentrations (Knol *et al.*, 1998). This type of interaction of Triton X-100 with bilayers has been confirmed in planar bilayers of PC using optical waveguide lightmode spectroscopy. As the free concentration of Triton X-100 increases, the detergent first incorporates reversibly into the bilayer. Subsequently, lipid molecules are partly removed from the bilayer and only when the free concentration in solution reaches the CMC (R_{sol}) the bilayers are solubilized (Csucs and Ramsden, 1998).

During reconstitution, ternary complexes of lipid, detergent and membrane protein can insert into detergent saturated bilayers. Triton X-100 molecules (in the bilayer and around the protein) catalyze the

insertion of the solubilized membrane protein, and, when the Triton X-100 concentration is lowered with hydrophobic beads, the equilibrium is shifted more and more towards protein insertion. The protein insertion is improved by mixing the liposomes and protein at 20 ^{o}C rather than 4 ^{o}C, which might be related to an increased fluidity of the lipid bilayer at higher temperatures and/or a change in the micellar size (Knol *et al.,* 1998; Hjelmeland, 1980).

Starting the reconstitution from the highest concentrations of detergent, with only mixed micelles present, the addition of the polystyrene beads (rapidly) leads to the formation of detergent saturated bilayers into which the protein inserts. The presence of Triton X-100-saturated membranes at different lipid to detergent ratios (between R_{sat} and R_{sol}) allows proteins to be inserted into bilayer structures over a broad range of detergent to lipid ratios without significant differences in specific activity. These observations have been made for several membrane proteins, including F_0F_1-ATPase from chloroplasts (Richard *et al.,* 1990), the glutamate carrier from *B. stearothermophilus* (Gaillard *et al.,* 1996), the lactose transport protein from *S. thermophilus* (Knol *et al.,* 1996), and the erythrocyte membrane anion exchanger, band 3 (Boulter *et al.,* 1996).

DODECYLMALTOSIDE

When liposomes are titrated with DDM, the equilibration of detergent is slow and at least 3 h are needed to achieve a constant OD (Lambert *et al.,* 1998, Knol *et al.,* 1998). Partitioning of DDM into the bilayer is more rapid in the presence of micelles than with detergent monomers alone. When DDM is added to liposomes in the monomeric form, the equilibration is non-cooperative and the process is followed (very slowly: > 1 week) by cooperative binding and membrane solubilization (Kragh-Hansen *et al.,* 1998). Detergents, like DDM, with strongly hydrophilic head groups and the shape of inverted cones only very slowly solubilize liposomal membranes and do not cause measurable liposome fusion. Liposomal structures are in case of DDM titration only found until the "onset of solubilization" (R_{sat}). In this first phase of liposome solubilization, the optical density initially decreases and subsequently increases reaching its maximum at R_{sat}. There are several explanations for the changes in OD_{540}, like changes in the average liposome diameter, the particle number, or the physical properties of the solution. The initial decrease indicates that liposome aggregates are dissolved by low concentrations of DDM. The increase in optical density at slightly higher concentrations can partly be explained by partitioning of detergent molecules in the bilayer and a subsequent increase in the diameter of the liposomes, but the structures formed are much more complex than that of a liposome. At R_{sat}, when the free concentration of detergent in solution is reaching the CMC, a large fraction of the liposomes has lost its integrity and the membranes are present as sheets (Knol *et al.,* 1998; Lambert *et al.,* 1998). The formation of these nonclosed bilayer sheets has been attributed to pore formation and hydrophobic shielding of the edges of the bilayer with a rim of detergent (Fromherz *et al.,* 1986; Lasic, 1988). When slowly more detergent is added, the structures convert into long thread-like micelles which gives rise to large increases in OD_{540}. The highest maximal OD values are obtained when the detergent is added in small amounts in a stepwise manner (Knol *et al.,* 1996). This increase has often been interpreted falsely as an increase in the diameter of the liposomes (De la Maza and Parra, 1997; Kragh-Hansen *et al.,* 1998). The consistency of the suspension can change dramatically at this phase and at lipid concentrations above 2 mM the suspension can even be "gel-like", implying the presence of highly entangled structures (Lambert *et al.,* 1998). This "gel-like" phase is observed with liposomes made of eggPC and eggPA (9:1), but not readily with liposomes composed of *E. coli* lipids (mainly PE) and eggPC (3:1) (unpublished results). This indicates that the nature of the structures formed are not only determined by DDM but also by the lipid composition of the bilayer. When vesicles derived from sarcoplasmic reticulum are solubilized by DDM, large structures are also formed, but these structures are less numerous and consist of smaller threads than present in the "gel-like" phase,

indicating an effect of for example the proteins on the formation and/or stability of the thread-like micelles (Kragh-Hansen *et al.*, 1993; Lambert *et al.*, 1998). At R_{sol}, large structures are no longer present and all the lipid and detergent molecules are present in small threadlike or spheroidal micelles (Vinson *et al.*, 1989, Knol *et al.*, 1998).

DDM is characterized by an hydrophobic tail of intermediate length and a bulky hydrophilic headgroup ("inverted cone" with critical packing parameter < ½), and the alignment of these molecules at saturating concentrations might cause curvature of the membrane and disruption of the bilayer structure. Unlike Triton X-100, the physical properties of DDM therefor support the formation of long micellar threads at intermediate detergent/lipid ratios (Phase II). Membrane proteins will not incorporate into such threads since they do not represent bilayer but rather micellar structures (Fig 2). It seems likely that membrane proteins will only be incorporated into the membrane sheets that are formed upon the removal of detergent to R_{sat}, that is when the stretched micelles are converted via lipid bilayer sheets into liposomes. When a protein is added to mixed micelles of lipids and DDM, it takes relatively long before the sheets are formed. Activity will be lost when the detergent solubilized protein is not fully stable and/or has the tendency to aggregate (Groth and Walker, 1996; Knol *et al.*, 1996). Aggregation of membrane proteins during reconstitution also depends on the efficiency of detergent removal from binary micelles (lipid and detergent) as compared to tertiary micelles (protein, lipid and detergent). If detergent molecules are more readily removed from lipid/detergent micelles than from the membrane protein, bilayers will form without the insertion of protein. When the detergent is removed from the hydrophobic surface of the membrane protein at a point that is beyond protein insertion this will lead to protein denaturation and/or aggregation. These features are important when other detergents are used, and in particular when a protein is reconstituted via rapid detergent removal.

OTHER DETERGENTS

Major structural changes of liposomes have also been reported for mixtures of lipids and other detergents, and the bilayer structures obtained with Triton X-100 may not be typical for the majority of detergents. Macromolecular structures distinct from those observed with Triton X-100 are observed when octylglucoside is added to PC vesicles. At detergent concentrations corresponding to R_{sat}, open vesicles co-exist with pieces of lamellae and long cylindrical micelles. At higher octylglucoside concentrations, the appearance of small spheroidal micelles, co-existing with cylindrical micelles, correlates with a decrease in OD (Vinson *et al.*, 1989; Wenk *et al.*, 1997).

Sheets of lipids have been observed by cryo-TEM for liposomes treated with cholate (Lasch, 1995), alkyl sulfate surfactants (Silvander *et al.*, 1996), octylglucoside (Ollivon *et al.*, 1988; Vinson *et al.*, 1989), and DDM (Knol *et al.*, 1998; Lambert *et al.*, 1998). In general, the bilayer sheets are formed as intermediates in the transition of vesicles to micelles, and they follow the formation of large detergent-stabilized pores. This is probably caused by a local increase in the curvature of the liposomal bilayer as a consequence of the incorporation of cone shaped molecules into the membrane. Open vesicles (or sheets) are stabilized by shielding of the free edges of the hydrocarbon chains by detergent molecules. Thread-like micelles have not only been reported for liposomes composed of egg PC plus PA treated with DDM, but also for mixtures of phospholipids with octylglucoside and phospholipids with alkyl sulfate surfactants (Vinson *et al.*, 1989; Almog *et al.*, 1990; Walter *et al.*, 1991; Edwards *et al.*, 1993; Silvander *et al.*, 1996; Knol *et al.*, 1998; Lambert *et al.*, 1998). Significant increases in dynamic light scattering at specific $C_{12}E_8$ to lipid ratios have also been interpreted as large structures of lipid and detergent in the form of sheets and/or threads (Edwards *et al.*, 1989; Edwards and Almgren, 1991; Kragh-Hansen *et al.*, 1998).

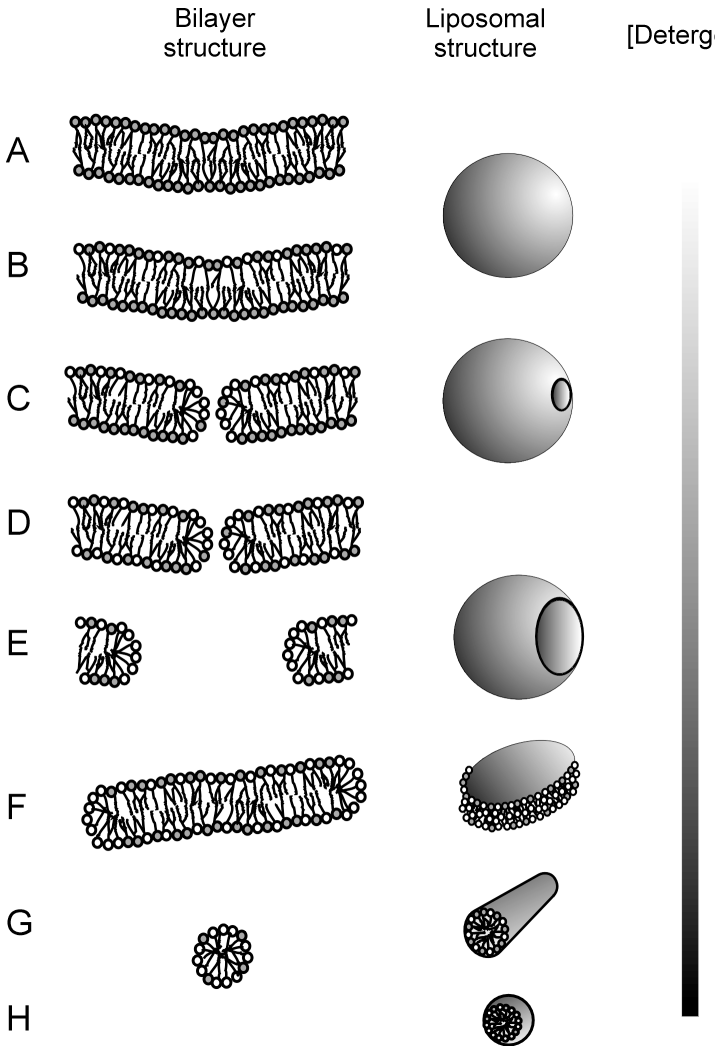


Figure 4. Model for the solubilization of lipid bilayers by DDM. *White circles indicate detergent molecules and gray circles indicate lipid molecules. For details see text.*

Summarizing the observations on the macromolecular structures of liposomes treated with detergent, we propose a model for the mechanism of membrane solubilization by detergents that are able to form thread like micelles (Fig. 4). When liposomes (A) are slowly (stepwise) solubilized, detergent molecules partition primarily into the outer leaflet of the bilayer (B). At a certain concentration, the inverted cone-shaped detergent molecules disrupt the bilayer structure locally and pores are formed. The edges of the "open liposomes" consist of detergent molecules, which shield the hydrophobic core of the membrane from the hydrophilic environment (C). These pores form sites at which detergent molecules can equilibrate rapidly with the inner leaflet, because the hydrophilic headgroups do not have to cross the hydrophobic core of the bilayer (D). Once these sites are formed, additional detergent molecules are more easily inserted, explaining the cooperative insertion of detergent molecules (Kragh-Hansen *et al.*, 1998). Detergent micelles (or membrane proteins covered with detergent in case of reconstitution) fuse with the rim of the bilayer which is covered by detergent (E). The increasing pore sizes will finally result in bilayer sheets with a rim of detergent molecules. At increasing detergent ratios the surface area of the sheet will decrease relatively to the edge, and transform from a circular sheet into more elongated structures. This is probable the stage at which membrane proteins should be added for optimal insertion, which might very well occur at the edges by fusion of the detergent rim with the protein molecule surrounded by detergent (F). At a critical ratio of lipids and detergent, the energetically most favourable structures are that of long micellar threads; these structures are induced by the inverted cones of the detergent molecules (G). The treads dissolve upon a further increase of detergent concentration, which allows the release of spherical mixed micelles (H). The thread-like structures (rods) do not allow the insertion of solubilized membrane proteins because, they do not have the essential bilayer structure.

In conclusion, the partitioning of detergent molecules in lipid bilayers, the molecular shape of the (detergent) molecules, and the flip-flop rates in the bilayer are critical parameters in the formation of macromolecular structures, and, consequently, the process of membrane reconstitution. These parameters explain many of the differences between detergents in membrane solubilization, and they provide a rationale for the mechanism of membrane protein reconstitution upon the removal of detergent.

ORIENTATION OF THE RECONSTITUTED PROTEIN

In the case of cytochrome *c* oxidase, bacteriorhodopsin, H^+-ATPase, Ca^{2+}-ATPase and other proteins, it has been observed that a more uniform orientation is obtained when the proteins are reconstituted into preformed liposomes treated with detergent (Eytan, 1982; Richard *et al.*, 1990; Levy *et al.*, 1992; Rigaud and Pitard, 1995). When a membrane protein is incorporated into preformed liposomes, the most hydrophilic domain will be least efficient in crossing the bilayer and the protein will insert with its most hydrophobic side first. The unidirectional orientation of the protein in the proteoliposomes can have clear advantages for the activity and the further (kinetic) characterization of the protein.

The differences in lipid-detergent structures observed with Triton X-100 and DDM provide a rationale for the unidirectional and random orientation of the LacS protein of *S. thermophilus* when the respective detergents are used to mediate the reconstitution (Knol *et al.*, 1996, 1998). In case of the LacS protein, the insertion is inside out when Triton X-100 is used to mediate the reconstitution, most likely because a relatively large hydrophilic domain is present at the cytoplasmic surface of the protein (Poolman *et al.*, 1989, 1995). Liposomes that are initially formed from mixed micelles of lipid and Triton X-100 (R_{sol}) will be small in diameter and the small radius of curvature of the liposomes results in packing defects of lipids. Lipids with an inverted cone shape will preferably partition into the outer leaflet, resulting in a lipid asymmetry which might favor the unidirectional orientation even more (Huang and Mason, 1978).

When DDM is used the LacS protein does not insert into 'intact' vesicles, but into lipid bilayers sheets or open liposome structures that are present during the conversion from micellar threads to liposomes (Fig. 4). The protein inserts from both sides, or at the edges of the bilayer in both orientations, and the orientation in the proteoliposomes is random (Knol *et al.*, 1998). A random orientation of the membrane reconstitution has been observed for several membrane proteins and detergent combinations, like bovine heart cytochrome *c* oxidase reconstituted by cholate dialysis; the sarcoplasmic reticulum Ca^{2+}-ATPase reconstituted by $C_{12}E_8$ removal using polystyrene beads; the kidney Na^+/K^+-ATPase reconstituted via deoxycholate dilution, and the *N*-acetylglucosamine transporter of *E. coli* reconstituted via octylglucoside dilution (Carroll and Racker, 1977; Levy *et al.*, 1992; Goldin, 1977; Mukhija and Erni, 1996). The orientation of the reconstituted proteins also depends on the speed of detergent removal. Reconstitution of H^+-ATPase by cholate dialysis (slow) is unidirectional, whereas cholate removal by dilution (fast) leads to a random orientation (Eytan, 1982). When the detergent is removed slowly, the formation of closed vesicles can apparently precede the (unidirectional) membrane insertion. When the detergent is removed rapidly, bilayer formation and insertion occur simultaneously, leading to a 50/50 distribution of the protein.

In vivo, all membrane proteins are inserted unidirectionally and cells use a protein machinery (Srp- and/or Sec-system) and metabolic energy (ATP and/or proton motive force) to obtain a correct insertion of membrane proteins (Bassilana and Gwizdek, 1996; Bochkareva *et al.*, 1996; Ulbrandt *et al.*, 1997). An important parameter for the orientation of insertion is the charge distribution of amino acid residues on both side of the membrane (Von Heijne, 1989). This charge distribution on the protein might also be important for the orientation of insertion into preformed liposomes.

There are indications that negative charges on acidic phospholipids are important for the initial binding of membrane proteins to lipid bilayers, and that the penetration of the protein is regulated by the type of acidic phospholipids. The insertion of rabbit microsomal cytochrome P450 into phospholipid bilayers has been measured by quenching of intrinsic Trp fluorescence by pyrene-labeled, brominated and doxyl-labeled phospholipids. When PC was replaced with acidic phospholipids (PA, PS, and PI) or PE, the extent of insertion is strictly dependent on the type of acidic phospholipids and this interaction might also play an important role in the orientation of insertion during the reconstitution (Ahn *et al.*, 1998). Also the orientation of cytochrome *c* oxidase in proteoliposomes prepared by cholate dialysis depends on the

electrostatic interactions between the protein and the phospholipids used. The right-side-out orientation of the protein when negatively charged phospholipids are used indicates that the positive charges on the protein might determine the orientation of the protein (Steverding *et al.*, 1989).

ASYMMETRIC KINETICS OF SECONDARY TRANSPORT PROTEINS

The unidirectional reconstitution of transport proteins into proteoliposomes allows characterization of the kinetic properties on either side of the membrane, that is binding to the inward and outward facing binding sites or translocation from out to in or *vice versa*. Although secondary transport proteins, such as the lactose transport system of *S. thermophilus*, operate reversibly in both directions, the kinetics of translocation via LacS are very different for opposite directions of transport. Proteoliposomes obtained via Triton X-100-mediated reconstitution yielded a unidirectional inside-out orientation of the protein with the affinity constants for lactose at the outer and inner surface of the membrane of ~0.2 mM and >10 mM, respectively. These values agree with what was known from *in vivo* studies, where the low affinity side of the protein is present at the outer surface. These studies clearly indicate that control of membrane reconstitution of this and other membrane proteins is crucial for a detailed kinetic analysis of their function. The more or less random orientation of LacS in proteoliposomes obtained with DDM explains in part the lower transport activity measured in the counterflow assay. In case of DDM-mediated reconstitution, about 50% of the molecules have the low affinity side exposed to the outside of the proteoliposomes. At substrate concentrations far below the K_m, about half of the molecules will thus not contribute significantly to the overall transport activity (Knol *et al.*, 1998).

LIPID TO PROTEIN RATIOS

A typical biological membrane can have a lipid to protein weight ratio of about 1. The reconstitution of purified membrane proteins, however, often requires much lower lipid to protein ratios. Cytochrome *c* oxidase from bovine heart, for example, can only be incorporated into liposomes at a lipid to protein ratio of 20 (w/w) or higher, and addition of more protein did not result in incorporation of more enzyme. However, addition of a different protein, the hydrophobic moiety of the H^+-ATPase, did lead to successful incorporation of more protein molecules (Eytan and Racker, 1977).

The activity of LacS from *S. thermophilus* also depends strongly on the final lipid to protein ratio and decreases rapidly at higher ratios. The strong dependence of specific transport activity on the concentration of LacS in the lipid membrane is not due to a change in the oligomeric state, but ESR observations on macroscopically aligned membranes shows structural heterogeneity in the protein reconstituted at lower lipid to protein ratios (Spooner *et al.*, submitted). The lipid to protein ratio was also found to be critical for the specific activity of the H^+-ATPase from chloroplasts (Richard *et al.*, 1990), the reconstitution of bacteriorhodopsin and H^+-transporting ATP-synthase from thermophilic *Bacillus* PS3 (TF_0F_1) (Pitard *et al.*, 1996), and the lactose transport system, LacY, of *E. coli* (Le Coultre *et al.*, 1998).

PROTEIN CRYSTALLIZATION

It is obvious that the characteristics of detergents and lipids are also relevant for 2D-crystallization of membrane proteins, which is an important alternative of 3D crystallization for the high resolution structure determination of membrane proteins (Henderson and Unwin, 1975; Engel *et al.*, 1992; Kühlbrandt, 1992; Ceska *et al.*, 1992; Kühlbrandt *et al.*, 1994; Wang *et al.*, 1994; Dolder *et al.*, 1996; Walz *et al.*, 1997; Walz and Grigorieff, 1998; Lacapere *et al.*, 1998). For stable arrays of protein and lipids,

Triton X-100 may be a 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 to shorter periods of detergent that may lead to inactivation and/or aggregation. Triton X-100 is also promoting the formation of bilayer membranes over a broad range of lipid to detergent ratios instead of aberrant structures like thread-like micelles (Saint *et al.*, 1998). The cylindrical molecular structure of this detergent might also be more appropriate to fill the space between protein molecules without causing a curvature of the growing crystal. On the other hand, the presence of bilayer sheets in case of detergents like DDM might allow growth of 2-D crystals at the detergent saturated edges and the random orientation might be advantageous for the formation of protein arrays. For a better understanding of the crystallization process using different detergents it is necessary to study the structures formed at lipid to protein ratios that are close to 1. Under these conditions, the protein will have a major impact on the macromolecular structures formed (Dolder *et al.*, 1996).

The structure of a number of membrane proteins have been determined by X-ray crystallography, e.g., bacterial light-harvesting complex, bacterial and mitochondrial cytochrome *c* oxidases, mitochondrial *bc*1 complex, α-hemolysin, bacterial porins and bacteriorhodopsin. These successes are partly based on advances in the crystallization procedures for integral membrane proteins, and it seems that variation of the size of the detergent micelle and/or increasing the size of the polar surface of the membrane protein represent important routes to obtain well-ordered membrane protein crystals (Rosenbusch, 1990; Ostermeier and Michel, 1997; Sakai and Tsukihara, 1998).

The use of bicontinuous lipidic cubic phases also appears to be promising for membrane protein crystallization. This membrane system, consisting of lipid, relatively low concentrations of detergent, water and protein, forms a structured, transparent and complex three-dimensional lipidic array, which is permeated by an aqueous channel system. Such matrices provide nucleation sites and support growth by lateral diffusion of protein molecules in the membrane. In this way crystals of bacteriorhodopsin were obtained that diffracted to atomic resolution (Landau and Rosenbusch, 1996; Pebay-Peyroula *et al.*, 1997). Proteins can even be fully active in these crystals as was shown for bacteriorhodopsin. Retinal isomerization, conformational changes in the protein backbone, and proton translocation are indistinguishable from those in the native membrane (Heberle *et al.*, 1998).