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Review

Membrane proteins, lipids and detergents: not just a soap opera

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Abstract

Studying membrane proteins represents a major challenge in protein biochemistry, with one of the major difficulties being the problems encountered when working outside the natural lipid environment. In vitro studies such as crystallization are reliant on the successful solubilization or reconstitution of membrane proteins, which generally involves the careful selection of solubilizing detergents and mixed lipid/detergent systems. This review will concentrate on the methods currently available for efficient reconstitution and solubilization of membrane proteins through the use of detergent micelles, mixed lipid/detergent micelles and bicelles or liposomes. We focus on the relevant molecular properties of the detergents and lipids that aid understanding of these processes. A significant barrier to membrane protein research is retaining the stability and function of the protein during solubilization, reconstitution and crystallization. We highlight some of the lessons learnt from studies of membrane protein folding in vitro and give an overview of the role that lipids can play in stabilizing the proteins.

Keywords: Membrane proteins; Lipid; Detergent; Bacteriorhodopsin; Reconstitution; Folding

Contents

Abbreviations: bR, bacteriorhodopsin; LCHII, light harvesting complex; DAGK, diacylglycerol kinase; DAG, diacylglycerol; SDS, sodium dodecyl sulfate; OG, *n*-octyl-β-D-glucopyranoside; DDM, *n*-dodecyl-β-D-maltoside; DDAO, dodecyldimethyl-*N*-amineoxide; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; DPPG, L-α-dipalmitoylphosphatidylcholine; DOPG, L-α-dioleoylphosphatidylglycerol; DOPC, L-α-1,2-dioleylphospatidylcholine; DOPE, L-α-1,2-dioleylphospatidylethanolamine; DHPC, L-α-1,2-dihexanoylphosphatidylcholine; DAG, diacylglycerol; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxyl-1-propanesulfonate; hR, halorhodopsin; OmpA, *E. coli* outer membrane protein A; OmpF, *E. coli* outer membrane protein F

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1. Introduction

Analysis of genomic sequence data predicts that 30% of the proteins produced by *Homo sapiens*, *Escherichia coli* and *Saccharomyces cerevisae* will be integral membrane proteins [1]. However, while the number of predicted gene sequences for integral membrane proteins has increased over the last few years, there is considerably less information about their three-dimensional structure and the nature of their behaviour within the membrane. There are several reasons for this dearth of information and we briefly outline some of these below before discussing approaches that can be used to address these problems.

1.1. Difficulties in the study of membrane proteins

The primary difficulty encountered in the study of membrane proteins is that of obtaining the protein of interest. Membrane proteins are usually present at low levels in biological membranes, and it is rare that a single protein species is a major peptidic constituent of the membrane. Where this is the case it has been exploited and a good example is bacteriorhodopsin (bR), which is the only protein present in the purple membrane of *Halobacteria salinaria* [2]. A straightforward preparation of the *H. salinaria* membrane is therefore sufficient to isolate high yields of pure protein. Another example is the predominant membrane transporter in red blood cells, the protein band 3, which has also been successfully studied [3]. Most membrane proteins, however, cannot be readily obtained in sufficient amounts from their native environments and thus attempts are made to overexpress them. A major problem with such heterologous expression of cloned constructs of membrane proteins, for example in *E. coli* or other systems, is aggregation of the protein in the cytoplasm. Thus, high yields of functional and stable protein are rarely obtained [2]. Moreover, mammalian proteins, such as the G protein-coupled receptor rhodopsin, also frequently require post-translational modifications that are unobtainable in bacterial hosts.

A second difficulty is that membrane proteins are naturally embedded in a mosaic lipid bilayer, which in even the simplest organism is a complex, heterogeneous and dynamic environment. This limits (but does not preclude) the use of many standard biophysical techniques to determine structure and function such as NMR, X-ray crystallography, circular dichroism, ligand-binding studies, classical kinetic characterization and the identification of structure–function relationships. Such biophysical methods are all but impossible to conduct in the native environment and are also frequently of restricted application since they require the protein to be extracted from its native membrane and studied in a detergent or lipid environment in vitro. This requirement leads to difficulties in sample preparation and spectral contributions from lipids.

Finally, membrane proteins are not generally soluble in aqueous solution. The need for membrane proteins to reside in surroundings that satisfy their high hydrophobicity therefore requires special synthetic systems for in vitro work. Unfortunately, reconstituting purified proteins into such systems has proven to be nontrivial.

Despite the inherent problems of working with membrane proteins, they remain an important area for study due to their role in the control of fundamental biochemical processes and their importance as pharmaceutical targets. To this end, many experimental methods have been devised to further the study of membrane proteins in vitro. This review will concentrate on the use of lipid and detergent systems in the reconstitution and crystallization of membrane proteins.

1.2. Strategies for studying membrane proteins

In light of the complexities of the lipid bilayer, it is highly desirable to transfer membrane proteins to a more

tractable environment for experimental study. Such systems will consist of a solubilizing component and must satisfy the hydrophobic nature of the transmembrane segments while bringing loop regions into contact with an aqueous phase. A number of approaches have been developed to meet these requirements and are currently used to solubilize and reconstitute membrane proteins in vitro. These systems may also aid in the crystallization of membrane proteins, something of a "holy grail" to those who work with them. We focus on the use of detergent micelles, mixed lipid/detergent micelles and bicelles, as well as liposomes for the reconstitution and crystallization of membrane proteins. Excellent comprehensive reviews have been published on these topics and the reader is directed to these for more detailed information on each topic. In this review, we give an overview of the methods currently available for solubilizing and reconstituting membrane proteins in the hope that this will provide a guide as to what can be achieved in this field. We assess the strengths and weaknesses of the methods and describe some of their applications. A summary of the methods and their applications is given in Table 1.

2. Detergents

The importance of detergents as tools for the study of membrane proteins cannot be underestimated. They are usually vital in the isolation and purification of the protein and are used in the primary solubilization step of reconstitution. They are also invaluable in membrane protein recrystallization. A comprehensive review of this area by le Maire et al. describes in detail the interactions of membrane proteins with detergents and provides an overview of techniques that may be employed for the structural investigation of detergent solubilized membrane proteins [4–6].

Table 1
Summary of different reconstitution methods for membrane proteins

Reconstitution method	Strengths	Weaknesses	Examples	References
Detergent micelles				
Ionic detergents, e.g. SDS	Excellent at solubilizing membrane proteins	Generally denaturing to some extent	bR, LCHII, DAGK, Ca^{2+}-ATPase	[7–11]
Bile acid salts	Mild and not generally deactivating.		Ca^{2+}-ATPase	[16]
Nonionic detergents, OG, DM, DDM	Mild and non-denaturing	Short chain can be deactivating	Ca^{2+}-ATPase,	[16–18]
Zwitterionic detergents	Uses in structural studies	Generally more deactivating than nonionic detergents	*Rhodopseudomonas sphaeroides* reaction centre, rhodopsin	[19,20]
Tripod amphiphiles	Shown to be successful at solubilizing certain proteins, removes need for traditional detergents	Limited use so far	Rhodopsin, bR	[24]
Amphipols	No micelles formed therefore less problems with viscosity or phase separation, removes need for traditional detergents	Limited use so far	Bacteriorhodopsin, reaction centre, OmpF, cytochrome b_6f	[25]
Lipid–detergent micelles				
Detergent solubilization	Facile incorporation of protein into the bilayer,	Protein must be stable in detergent, detergent removal must be chosen carefully to suit specific properties of detergent.	bR, LmrP, LacS,	[56–58]
Dilution	Good for detergents with high cmc's	Leaky proteosomes, inhomogeneity in protein distribution.	OmpF	[41]
Organic solvent mediated	Preparation of liposomes with a large internal volume	Exposure to organic solvent can denature membrane proteins, resulting liposomes often fragile	Rhodopsin, cytochrome *c* oxidase, acetyl choline receptor, bR	[59–64]
Sonication	Freeze thawing improves liposome quality—good technique for proteins that cannot withstand detergent.	Small liposomes—often deactivated protein	D-glucose carrier from red blood cells	[65]
Bicelles				
	May be a better mimic of the lipid bilayer. Useful in structural studies such as NMR	Limited application so far	DAGK	[33–35]

2.1. Types of detergent

Detergents are amphipathic molecules, consisting of a polar head group and a hydrophobic chain (or tail), and exhibit unique properties in aqueous solutions in which they spontaneously form (generally) spherical micellar structures. Membrane proteins are frequently soluble in micelles formed by amphiphillic detergents. Detergents solubilize membrane proteins by creating a mimic of the natural lipid bilayer environment normally inhabited by the protein.

2.2. Classification of detergents

Detergents are classified according to their structure and fall into four major categories. Here is given a brief overview of the classes of detergent and their effects on membrane proteins. Fig. 1 gives an example of each class of detergent.

Ionic detergents contain a head group with a net charge that can be either cationic or anionic (see Fig. 1a). They also contain a hydrophobic hydrocarbon chain or steroidal backbone. The *critical micelle concentration* (cmc) of an ionic detergent is determined by the combined effect of the head group repulsive forces and the hydrophobic interactions of the tails. Ionic detergents, such as sodium dodecyl sulfate (SDS), are extremely effective in the solubilization of membrane proteins but are almost always denaturing to some extent. Some proteins can be renatured from sodium dodecyl sulfate by transferring the protein to a renaturing detergent or lipid environment. Examples include bR [7], the major light harvesting complex of higher plants, LHCII [8,9] and *E. coli* diacylglycerolkinase (DAGK) [10]. Furthermore, it has recently been shown that it is possible to reactivate SDS denatured Ca^{2+}-ATPase (from sarcoplasmic reticulum) by ceramic hydroxyapatite chromatography, followed by exchange into an alternative mild detergent [11]. In these cases, SDS can be removed from the final mixed detergent/lipid mixture by organic solvent precipitation [12], ion-pairing reagents [13], precipitation as potassium dodecyl sulfate [14] or reversed phase HPLC [15], but in other cases removal of SDS can often lead to irreversible aggregation and precipitation of the protein.

Bile acid salts are ionic detergents, which differ from SDS in that their backbone consists of rigid steroidal groups (see Fig. 1b). As a result, these bile acid salts have a polar and apolar face, instead of a well-defined head group, and they form small kidney-shaped aggregates unlike the spherical micelles formed by traditional ionic linear-chain detergents. Bile acids are relatively mild detergents and are often less deactivating than linear-chain detergents with the same head group [16].

Nonionic detergents contain uncharged hydrophilic head groups of either polyoxyethylene or glycosidic groups (see Fig. 1c). Nonionic detergents are generally considered to be mild and relatively non-denaturing, as they break lipid–lipid interactions and lipid–protein interactions rather than protein–protein interactions. This allows many membrane proteins to be solubilized in nonionic detergents without affecting the protein's structural features, such that it can be

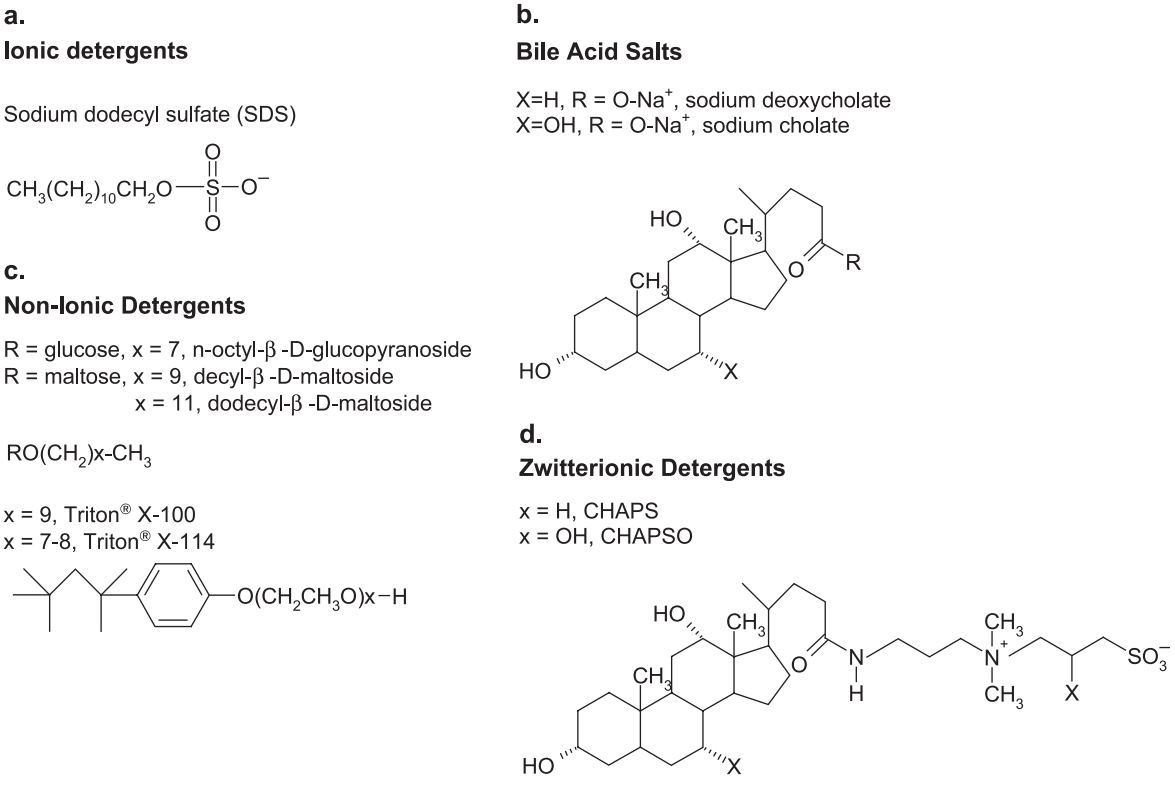

Fig. 1. Types of detergent.

isolated in its biologically active form. However, short chain (C_7–C_{10}) nonionic detergents, such as *n*-octyl-β-D-glucopyranoside (OG), can often lead to deactivation of the protein, unlike their corresponding intermediate (C_{12}–C_{14}) chain derivatives [16].

Other alkylglucosides, such as *n*-dodecyl-β-D-maltoside (DDM), are increasingly used in membrane protein solubilization as many proteins can be readily solubilized in a functional state in DDM but with retention of functional properties [16–18].

Zwitterionic detergents (see Fig. 1d) combine the properties of ionic and nonionic detergents and are in general more deactivating than nonionic detergents. They have, however, found uses in structural studies of membrane proteins. Examples include the use of dodecyldimethyl-*N*-amineoxide (DDAO) in the crystallization of the reaction centre of *Rhodopseudomonas sphaeroides* [19] and structural work on rhodopsin [20].

2.3. General properties of detergents

The choice of detergent for membrane proteins is influenced by the type of work to be carried out; however, there are properties that are common to all detergents that may be helpful in deciding which is the most suitable agent for the job in hand.

The cmc can be defined as the minimum concentration of detergent for individual detergent molecules to cluster and form micelles, such that there is a sudden change in surface tension and other physical properties. Above the cmc the detergent monomer concentration is also independent of the total detergent concentration. The cmc varies with conditions, including pH, ionic strength, temperature as well as the presence of protein, lipid and other detergent molecules ([4]). The cmc decreases with the length of the alkyl chain of the detergent and increases on the introduction of double bonds and branch points (for example, those present in bile salts, which consequently have a high cmc). In ionic detergents, the cmc is intrinsically linked to the counter-ion present—the cmc is reduced upon increasing the concentration of counterions. It will be seen later when discussing techniques for the removal of detergent that a high cmc is desirable if the detergent is to be removed by dialysis.

At low temperatures, detergents remain mainly in a crystalline insoluble form that is in equilibrium with small amounts of solubilized monomers. As the temperature is increased, more monomer dissolves until the cmc is reached; this is known as the *critical micellar temperature* (cmt). The temperature at which the crystalline form, monomer and micelles exist in equilibrium is known as the *Kraft Point*. In most cases, this will be equal to the cmt.

Above the cmt, nonionic detergents become cloudy and phase separate into a detergent-rich layer and an aqueous layer. The temperature at which this occurs is called the *cloud point*. A low cloud point can be advantageous in the solubilization of membrane proteins, for example, the nonionic detergent Triton X-114 has a cloud point of 22 °C, thus the protein can be solubilized at 0 °C and then brought to 30 °C to allow phase separation to occur. The membrane protein can then partition into the detergent phase, which can then be separated by centrifugation [21]. The number of monomers contained in a micelle is known as the *aggregation number*. It is calculated by dividing the relative molecular mass of the micelle (obtained by gel filtration, sedimentation equilibration or light scattering) by that of the monomer. Bile salts tend to have low, ionic strength-dependent aggregation numbers, where the aggregation number of nonionic detergents tends to be much higher.

2.4. Removal of detergents

An excess of detergent is employed to solubilize membrane proteins as this ensures complete dissolution of the protein. This excess detergent can unfortunately complicate spectra or disrupt further experimental work and so often must be removed once proteins are solubilized. Various removal methods exist to allow transfer of the membrane protein into a liposome or into a different detergent. These methods take advantage of the properties of the detergent in question, for example, the cmc, the charge or the aggregation number.

2.4.1. Dialysis

Dilution of detergent to concentration values below the cmc results in the disintegration of micelles to individual detergent monomers. The monomers are considerably smaller than the micelles, and as a result they can be easily removed by dialysis. Dialysis is the most common form of detergent removal and this process typically requires dialysing the protein detergent mixtures against detergent-free buffer (in about 200-fold excess) over a period of days. This technique is more practical with detergents with a high cmc and works best for those with low molecular weight/small cross-sectional area. The technique is unsuitable for detergents with a low cmc, for example, nonionic detergents.

2.4.2. Hydrophobic adsorption

Detergents, by virtue of their amphiphillic nature, can bind to insoluble hydrophobic resins or “beads”, through the interaction of their hydrophobic detergent tail with the hydrophobic surface of the bead. The detergent-containing solution is mixed with the resin and allowed to stand. The detergent-coated resin can then be removed by centrifugation or filtration. This method is especially suitable for the removal of detergents with a low cmc. Hydrophobic beads have been successfully employed for detergent removal to elucidate new 2D crystal structures for Ca^{2+}-ATPase, melibiose permease and cytochrome b_6f [22].

2.4.3. Gel chromatography

This technique exploits the size differences between protein–detergent and detergent micelles or detergent–lipid micelles. To prevent protein aggregation and precipitation, the elution buffer needs to contain a further detergent below its cmc. As this technique works on the basis of size separation, factors that may influence the size of the micelle should be kept constant, for example, pH or temperature.

2.4.4. Ion-exchange chromatography

Ion-exchange chromatography uses the difference in charge between protein–detergent micelles and homogeneous detergent vesicles. Using nonionic or zwitterionic detergents, it is possible to select conditions that retain the protein–detergent micelles on the column. The protein can then be eluted by a change in ionic strength or pH, or by washing with an ionic detergent.

2.4.5. Nickel columns and His tags

Nickel columns provide an efficient method of transferring proteins that are overexpressed with His tags into different detergent environments. The His-tagged protein in detergent micelles will bind to the column, and can be exchanged by washing the column with another detergent micellar solution prior to elution off the column.

2.5. Alternatives to traditional detergents

Detergents provide a convenient means of solubilizing and handling membrane proteins during purification as well as for many other methods including crystallization. However, in reality, detergent systems are a poor mimic of the native membrane environment in which such proteins are normally found. Although some membrane proteins are functional in detergent environments, detergents are frequently destabilizing and can lead to inactivation of the protein over time. Thus, efforts have been made to design new solubilizing agents that are less destabilizing than traditional detergents. To this end, a class of compounds known as *tripod amphiphiles* [23] has been used to a great degree of success in the solubilization of bacteriorhodopsin and bovine rhodopsin from purple membrane [24], keeping the protein in a monomeric native-like form for several weeks. Tripod amphiphiles consist of a tetrasubstituted carbon atom carrying three hydrophobic tails and a polar head group. These compounds are thought to limit the length and flexibility of the hydrophobic moieties, which may be implicated in membrane protein inactivation.

A completely new class of solubilizing agents have also been designed consisting of a mixed copolymer with a hydrophilic backbone and hydrophobic side chains. These *amphipols* [25] are thought to wrap around the hydrophobic portion of the protein and expose their hydrophilic backbone to the aqueous environment. Amphipols may have significant advantages over traditional detergents as, in any detergent-solubilized protein solution, there will be an amount of free detergent that can be present as monomers or micelles. This free detergent can lead to phase separation problems during crystallization, or a viscosity increase in NMR experiments [25]. As the amphipol is completely associated with the protein in a stable complex, there is little or no free polymer in solution, thus minimising these problems.

3. Mixed lipid–detergent systems

An ideal situation for in vitro membrane protein work would be to work in an environment that more closely resembles the natural lipid bilayer that surrounds the membrane protein in vivo. The complexity of the natural bilayer, however, means that recreating this exact environment is impossible. Nevertheless, with judicious use of lipids, a more suitable system than detergent alone can be designed for stabilizing membrane proteins. Indeed, a combination of detergent and lipids may often prove fruitful in NMR and crystallization experiments.

3.1. Detergent–lipid micelles and bicelles

Membrane proteins can also be purified into lipid/detergent micelles. In this system, the hydrophobic regions of the protein are solvated with the nonpolar groups available in a dispersed lipid solution. For example, the apoprotein of rhodopsin (opsin) is generally unstable in detergent solution. Stable opsin can be purified into micelles made of a mixture of the lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and the detergent 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS). These DMPC/CHAPS micelles have allowed the study of the kinetics of cofactor binding to opsin and formation of rhodopsin [26]. Bacteriorhodopsin can also be refolded from an SDS denatured state (where there is an α-helical content equivalent to about 3 to 4 helices) [27,28] into DMPC/CHAPS or DMPC/L-α-1,2-dihexanoylphosphatidylcholine (DHPC) micelles at a yield of ca. 95%. The refolding can be followed by protein fluorescence [29], retinal absorption [30] and protein circular dichroism [28]. Reversible refolding of bR in mixed DMPC/CHAPSO/SDS micelles has also been demonstrated [31]. LCHII can be denatured in SDS to form the apoprotein and subsequently refolded into OG micelles containing the pigments required for function [8,9,]. However, addition of lipid to the OG micelles increases the thermal stability of the folded state [32]. This effect is not specific to a particular lipid and is seen on refolding in OG micelles containing either native thylakoid lipids monogalactosyl diacylglycerol (MGDG) or digalactosyl diacylglycerol (DGDG) or the synthetic lipids L-α-dipalmitoylphosphatidylcholine (DPPG) or L-α-dioleoylphosphatidylglycerol (DOPG).

When certain detergents such as DHPC or CHAPS are mixed with short chain lipids such as DMPC in the correct

composition and at the correct temperature, bilayered discoidal structures known as *bicelles* may be formed [33]. Bicelles have a much lower detergent concentration than traditional mixed micelles and have some bilayer characteristics that make them potentially more useful as an environment within which to solubilize functional membrane proteins. Bicelles can also be magnetically aligned, thus lending them to NMR studies. Membrane proteins can be reconstituted in functional form within the bicelle as was demonstrated with DAGK in a range of bicelles based on varying-chain-length PC lipids with the bile salt detergent, CHAPSO. However, the activity of DAGK in these bicelles was lower than that of the protein in mixed micelles or vesicles. Work is ongoing to improve the lipid to detergent ratio that will yield optimal activity and to discover novel bicellar systems [34,35]. Bicelles also have applications in the field of membrane protein crystallography (see later).

3.2. Reconstituting proteins into bilayers

Phospholipid vesicles that contain membrane proteins are known as *proteoliposomes* and are an excellent tool for the elucidation of membrane protein structure and function. There are a number of criteria that the proteoliposomes should fulfill for them to be considered useful as systems for functional studies. A homogeneous size distribution of proteoliposomes is often required, and can be achieved by extrusion (filtration under high pressure) through a polycarbonate membrane of selected size. It is also useful if protein is evenly distributed among the liposomes [36,37]. The biological activity of the embedded membrane protein in the proteoliposomes must remain high [36,38] and it is helpful if protein reconstitution is efficient over a variety of lipid to protein ratios [37]. The membrane permeability of the proteoliposome to counter-ions or unwanted proton transport must also be low [38,39]. If the study concerns transport activity, or other events that are dependent on protein directionality and topology, then all proteins must be inserted in a single transmembrane orientation to ensure that pumping of substrates occurs in the correct direction, whether this be inside-out or outside-in [40]. The reconstitution of bR into liposomes fulfills these criteria well and has shown to be useful as a model system.

There are a variety of methods to insert membrane proteins into liposomes. Reconstituting proteoliposomes via a detergent-mediated pathway is often successful, and is convenient since detergent is usually used in the initial isolation and purification of the protein. An excellent review of the formation of liposomes and proteoliposomes, together with an overview of the reconstitution of energy-transducing membrane proteins, has been given by Rigaud et al. [41]. There are two common approaches. The first is a simple dilution approach. This essentially supposes that if a protein–detergent mixture is diluted into a liposome solution, such that the concentration of detergent falls below the cmc, the detergent micelle becomes unstable and the protein transfers into the liposome. The second approach involves introducing detergent to preformed liposomes such that the liposome bilayer becomes saturated with detergent. The detergent disrupts lipid–lipid interactions, which results in a more permeable bilayer. These saturated structures are more receptive to protein uptake, and after protein is introduced the excess detergent can be removed by several methods (dialysis, column chromatography or incubation with detergent-adsorbing beads), as discussed previously. The method of choice will depend on the physico-chemical properties of the detergent selected.

3.3. Detergent solubilization of liposomes

The transformation between liposomes and detergent/lipid mixed micelles is a reversible process that can be induced by the addition or removal of detergent from the starting solution. The process takes place according to a three-stage model that has been elucidated by a wide variety of experimental techniques, for example turbidimetry [42–45], fluorescence energy transfer [46,47], magnetic resonance spectroscopy [48–50], quasi electric light scattering [51,52], centrifugation [42,45] and electron microscopy [53,54]; the most widely applied of which has been turbidimetry. There is a dramatic decrease in turbidity of a sample associated with the transition from vesicular to micellar states which can be used to follow the solubilization process. For an overview of the interactions of detergents with phospholipid vesicles, see Inoue [55] and references cited therein.

Fig. 2 illustrates the phase transition from liposome to micelle caused by detergent partitioning into bilayers. During the first stage, detergents are added to the preformed vesicle solution. These partition into the vesicle membrane, until a saturation point is achieved. This leads to a breakpoint in the turbidity curve (Fig. 2, line 1). Further addition of detergent to the already saturated vesicles leads to destruction of the vesicles and the solution now consists of detergent-saturated vesicles and lipid-saturated mixed micelles. Eventually, the system will consist only of mixed micelles (Fig. 2, after line 2). Any further addition of detergent will lead merely to the dilution of the phospholipid within the micelle.

The detergent-saturated liposomes seem to favour the partitioning of membrane proteins from solution. Excess detergent can then be removed to generate functional proteoliposomes. This approach has been exploited in the reconstitution of several transport proteins from detergent states. Also, bR has been successfully incorporated into liposomes by detergent mediation, with the type of detergent used playing a large part in the mechanism of protein insertion. When sodium cholate was the detergent used, proteoliposomes were only formed from ternary mixed micelles of lipid, protein and detergent. However,

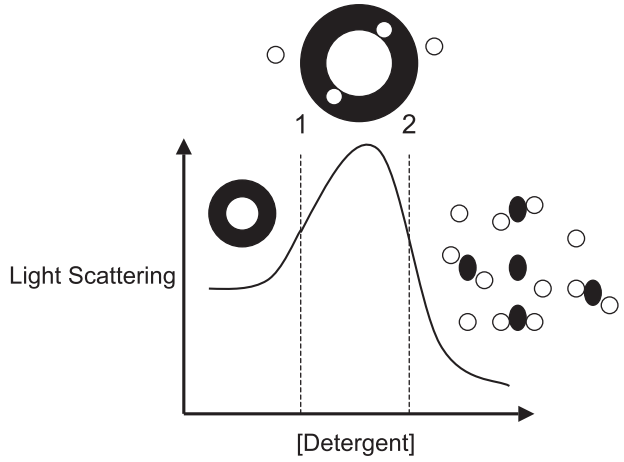

Fig. 2. Schematic diagram showing the phase transition from liposome to micelle caused by detergent partitioning into bilayers. Filled black regions represent lipids or liposomes; open white circles are detergent molecules. The solid line is an indication of the change in turbidity of the sample at various stages, which can be analysed by following changes in light scattering. Dotted lines estimate phase transition. Prior to line 1, detergent begins to partition into liposome bilayers. Between lines one and two, detergent concentration approaches saturating values and large, saturated liposomes are formed. After line two, the majority of liposomes is broken down into mixed micelles, eventually reaching total solubilization. The process is reversible. The ratios of detergent to lipid at the point of greatest saturation (R_{sat}) and total solubilization (R_{sol}) can be thus be determined through light scattering measurements.

when OG was used, direct incorporation of bR into detergent saturated liposomes occurred and optimal proton pumping activity was achieved [56]. The multidrug transporter LmrP has also been successfully reconstituted by this method. The protein was solubilized by DDM, then reconstituted into liposomes of *E. coli* lipids that had previously been saturated and destabilized by DDM [57]. The detergent was subsequently removed with detergent adsorbing Biobeads. This method proved successful after other detergents such as Triton X-100 and X-114 and Tween 80, which were however shown to inhibit LmrP-mediated H+/drug antiport activity. Reconstitution of the lactose symporter of *S. thermophilus* into liposomes destabilized by DDM or Triton-X has also been investigated, but with strikingly different results in terms of stability of the liposomes and the orientation of the protein within the liposomes. DDM was found to disrupt the membrane structure of liposomes formed from egg yolk phosphocholine and *E. coli* lipids at the onset of solubilization and threadlike micelles were observed at higher detergent to lipid ratios [58]. The protein inserted into these liposomes in a random orientation. This is in stark contrast to the results found with the same protein reconstituted into liposomes destabilized by Triton-X which showed all the protein hydrophilic surfaces exposed to the outside of the liposome and maximal transport activity in liposomes of this type. Recent results from our laboratory suggest that the saturation method is applicable to other transport proteins such as the small multidrug resistance protein EmrE (P. Curnow, M. Lorch and P.J. Booth, unpublished data).

3.4. Other reconstitution methods

Organic solvent-mediated reconstitution has been used to prepare liposomes with a large internal volume [59–61]; however, the exposure of membrane proteins to organic solvents often leads to their denaturation. Evaporation of a solution of a protein–lipid complex in an apolar solvent followed by rehydration and sedimentation through sucrose has been shown to lead to giant proteoliposomes containing active proteins such as rhodopsin, cytochrome *c* oxidase, reaction centre and acetylcholine receptor [62]. However, these liposomes did not allow for detailed functional study of the proteins due to their fragility. Reverse-phase evaporation techniques have been successfully used for the incorporation of rhodopsin [63] and bacteriorhodopsin [64]. Large unilamellar proteoliposomes were formed from a (water in oil) emulsion of lipid, protein and buffer in organic solvent (such as pentane, hexane or diisopropyl ether) followed by removal of the organic phase under reduced pressure.

It is possible to affect the transfer of proteins into liposomes by sonication of a mixed suspension of the protein in buffer and lipid of interest. However, the small liposomes produced by sonication can cause the protein to become inactive [41]. This technique can be greatly improved by freeze-thawing of sonicated liposomes mixed with protein, followed by a further sonication step [65]. This technique allows the rapid production of proteoliposomes and can be used for proteins that cannot withstand detergent or are sensitive to sonication.

3.5. The effects of lipids on protein stability and folding

While examination of membrane proteins in their native environment is nontrivial due to the heterogeneous nature of the lipid bilayer, the effects of membrane lipids on the functionality, stability and folding of membrane proteins are a rapidly expanding area of research. The denaturation and subsequent refolding of water-soluble proteins has provided many insights into the determinants of the structure and function of these proteins [66]. Similar information on membrane proteins would be of considerable interest, yet technical difficulties mean that very few membrane proteins have been reconstituted from states of structural deformation and nonfunctionality.

Currently, only Bacteriorhodopsin [27,67], Light Harvesting Complex II [8], *E. coli* Diacylglycerol kinase [68] and outer membrane proteins OmpA [69] and OmpF [70] have been reconstituted into liposomes from partially denatured states. Data on these proteins have allowed thermodynamic and kinetic evaluation of events leading to formation of the native state in the bilayer. In addition to the direct research interests associated with the field, there is a

more prosaic interest in such work since the detergent-soluble inclusion bodies that frequently occur during heterologous membrane protein expression [2,71] are difficult to solubilize and refold correctly.

Native biological membranes do not consist of a single component and are frequently made up of a combination of "bilayer" and "non-bilayer" forming lipids. These different properties of the lipids impart particular characteristics on membranes that can be mimicked in vitro with simple combinations of synthetic lipids. Commonly used lipids include 1,2-dioleoyl-*sn*-glycerophosphocholine (DOPC), which is a bilayer lipid that forms fluid lamellar bilayers under physiological and laboratory conditions, and 1,2-dioleoyl-*sn*-glycerophosphoethanolamine (DOPE), which is a non-bilayer lipid that will adopt non-lamellar reverse hexagonal (H_{II}) structures [72,73]. The presence of a non-bilayer forming lipid increases the propensity of the polar–apolar face of each monolayer to curve towards water. However, the hydrophobic effect prevents monolayers from separating, and so a torque stress is induced into the membrane. This is accompanied by an increase in the lateral pressure in the centre of the bilayer due to the increase in the number of collisions between hydrocarbon chains. Tighter packing in head-group region leads to more intermolecular collisions in chain region.

By careful consideration of the lipids used to create liposomes within which to study proteins, the effects of lateral pressure within the bilayer on the insertion and folding steps of the protein can be elucidated. An increase in lateral pressure can be achieved by introducing lipids of the same head group type but of differing chain lengths and has been shown to slow the rate-limiting folding step in bR by approximately 10-fold [74,75]. However, lipid chains of different lengths can lead to different hydrophobic thicknesses within the bilayer, which can in turn lead to differences in the lateral pressure. Using mixed DOPC/DOPE bilayers, changes in curvature and stress have been shown to affect bilayer partitioning of the voltage-gated ion channel Alamethicin [76], the activity of the G protein-coupled vision receptor rhodopsin [77], the activity of the PC biosynthesis enzyme CCT [78] and the activation energy for spontaneous insertion of helical peptides [79]. Bilayer tension also appears to play a role in the conformational stability and activity of Gramicidin A [80,81].

E. coli DAGK phosphorylates DAG and is important in lipid biosynthesis as well as removing toxic DAG from the membrane, and it has been successfully refolded from a urea or guanidinium denaturant solution into POPC vesicles and mixed detergent/lipid micelles consisting of DDM and cardiolipin. The rates and efficiencies of insertion and folding were monitored and compared with those gained from the dilution of micellar DAGK solutions into POPC vesicles [82]. The rate was observed to decrease when the protein was inserted into vesicles compared to insertion into mixed detergent lipid micelles, suggesting that the rate-limiting step for DAGK assembly may be the protein entering the lipid bilayer. Reversible unfolding of DAGK has also been demonstrated in detergent micelles consisting of DM and SDS [10].

3.6. Specific lipid interactions

Crystallography of membrane proteins has also provided an insight into the roles of specific membrane lipids in key biophysical functions within the protein–lipid environment [83,84]. Crystallization of the reaction centre of *Rb. sphaeroides* showed the presence of a cardiolipin lipid molecule located on the intramembrane surface [85]. This cardiolipin is also included in a number of other structures for mutant *Rb. sphaeroides* reaction centres [86]. While there has been no study of the relevance of this lipid to the structure and function of the reaction centre, it is known that proteins containing bacteriochlorophyll from purple bacteria have a preferential association with negatively charged lipids (including cardiolipin) [87,88]. Moreover, the residues that bind the head-group of the cardiolipin are strongly conserved across a number of purple photosynthetic bacteria [89], which suggests a specific role for this protein–lipid interaction within the complex.

Cardiolipin molecules have also been resolved in the structure of bovine cytochrome *c* oxidase [90] where they are essential for the protein's function, with removal of the lipid leading to loss of enzymatic activity [91,92]; again, however, there is no information on the specific role played by this lipid. The thermal steps of the photochemical cycle of bR are also affected by the presence of specific lipids; in particular, a combination of squalene and phosphatidyl glycerophosphate is required to maintain normal photochemical behaviour. It is clear that while the role that membrane lipids play in membrane protein structure and function is beginning to be understood, there is still a gap in our knowledge of the complexity of the specific interactions within protein–lipid complexes.

4. Crystallization of membrane proteins

Membrane proteins are fundamental to many aspects of biology and thus knowledge of the protein structure at near atomic level resolution is a critical step towards a molecular level understanding of membrane function. The major barrier to obtaining structures of membrane proteins is the preparation of diffraction quality crystals. A variety of techniques exist to produce suitable three dimensional crystals; these are based on detergent ("in surfo") and lipid ("in cubo") systems or on the use of lipid bicelles. Comprehensive reviews have been published on all of these methods [92,93]; this paper aims to provide an overview of the salient features with relation to the uses of detergents and lipid membrane protein crystallization.

4.1. In surfo method

The basis of the in surfo method is the incorporation of protein (and any residual lipid that may be present) into detergent micelles. The production of crystals can then occur via standard routes such as vapour diffusion or microdialysis. It has been suggested that the inherent problems with the in surfo method lie in the protein flexibility and conformational inhomogeneity. There may also be a lack of protein–protein contacts within the crystal, due to the presence of the surfactant that surrounds the protein. The addition of antibody fragments can go some way to alleviate this problem and stabilize protein–protein contacts while also minimising flexibility in the protein–antibody co-crystal [94]. There are detailed reviews that provide a comprehensive overview of the in surfo method [95,96] and, to date, this approach has been successfully employed in the crystallization of cytochrome *c* oxidase, cytochrome b_1c and the KcsA potassium channel [94].

4.2. In cubo method

The in cubo method of crystallization works on the basis that a membrane protein should crystallize with greater ease in an environment which is similar to its natural bilayer. Lipids can self-assemble into various mesophases; the planar lipid bilayer is one such state. However, they can adopt non-lamellar phases such as inverse hexagonal or cubic phases. Which phase is present will depend on the temperature, the lipid in question and the concentration. The in cubo method is based on a bicontinuous cubic phase composed of monoacylglycerols (for example monoolein) and water [97]. The cubic phase is prepared by mixing (usually) monoolein to an aqueous dispersion of protein at a given ratio; the cubic phase forms spontaneously. Salts can then be overlayed on the cubic phase, either as solids or in solution, which facilitates protein precipitation. The sample is then incubated until crystals form, which can take from hours to weeks.

The first protein to be successfully crystallized by this method was bR [97,98], to a resolution of 1.55 Å in a monoolein lipid cubic phase [99–102]. Not only did this show an improvement in the previous resolution of bR crystals, but structures of some of the intermediate states involved in the bR photocycle could be elucidated [103–105]. bR was successfully crystallized directly from its native purple membrane [106], which avoided the need for exposure of the protein to detergents; this fact is significant in the consideration of the crystallization of proteins that are unstable in the presence of detergent. This in cubo method has since been shown to be suitable for the crystallization of a variety of membrane proteins—the photosynthetic reaction centres from *Rb. sphaeroides*, *Rb. viridis*, light harvesting complex II from *Rhodopseudomonas acidophila* and halorhodopsin from *H. salinarium* (hR) [107]. However, so far all the proteins that have had their structures determined by this method have contained chromophores. There is no reason to imagine that non-coloured proteins cannot be crystallized in cubo, but detection of the crystals in the lipid matrix is more challenging and the presence of precipitant salt crystals may exacerbate this problem. Work is ongoing to develop a simple solution, for example by staining the crystals, labelling with a dye [108] or genetic modification of the protein to include a coloured domain [109–111]. An excellent overview on the behaviour of monoolein with various detergents can be found in the work of Sennoga et al. [112]. The implications for crystallization at low temperatures are also discussed.

4.3. Bicelle method

This approach to protein crystallization applies a similar methodology to both the in surfo and in cubo methods, but uses the discoidal micelles of lipid and detergent discussed previously. Crystals of bR [113] have been grown by combining a bicelle solution of DMPC and CHAPSO with bR (as purple membrane) on ice, to ensure that the mixture was in its liquid phase. The mixture was then incubated at 37 °C at which time a colourless, viscous gel was formed. Crystals were harvested at room temperature (meaning that the suspending medium was now in its liquid phase). The bR crystallized as a new form, which was found to be highly delipidated and contained enhanced protein–protein contacts within the crystal. To date, bR is the only protein that has been successfully crystallized by this method, but it may hold much promise for the future.

5. Conclusions

Membrane proteins are arguably one of the most challenging areas of the proteosome, and remain one of the most under studied. Integral membrane proteins make up a significant proportion of the proteosome in many organisms and play a vital role in a myriad of diverse cell functions including signalling, energy generation, transport and recognition. They also remain of considerable significance as potential targets for pharmaceuticals. To the casual observer, it is therefore perhaps surprising that we know so little molecular level detail regarding their structure and function. Less than 1% of structures deposited within the Protein Data Bank are membrane proteins and mechanistic information is available for a bare handful of these. As discussed above, in vitro studies appear to be the only means to derive this information. The synthetic systems necessary for such in vitro studies are currently poorly understood. Selecting and implementing the correct systems is crucial to the success of membrane protein studies, and can be a painstaking trial-and-error process. Some membrane proteins are soluble only in a single detergent species that fulfills specific solubilization requirements; others are soluble in many different detergents but are only functionally active in one of them. An understanding of the

detergent parameters that determine solubility and functionality will be crucial to the continued understanding of integral membrane proteins. However, much has been learned thus far about the techniques that can be applied to this area. It has been amply demonstrated that by careful consideration of detergents, lipids and reaction conditions, the techniques that seem trivial when applied to water-soluble proteins can also find their uses in the elucidation of the structure and functionality of membrane proteins.

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