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Review

Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins

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Contents

1. Introduction

Studies of membrane proteins in their native environment can be difficult to interpret due to restrictions arising

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from the complexity of the native membranes and interferences with other membrane constituents or other reactions. In membrane research, phospholipid vesicles incorporating purified membrane proteins (proteoliposomes) are therefore a powerful tool for elucidating both functional and structural aspects of these membrane-associated proteins. This approach has been applied with success to a very diverse range of membrane functions. Within this framework, membrane reconstitution has played a central role in the studies of membrane proteins with a vectorial function (transport) and in particular has helped to improve our understanding of the mode of operation of energy-transducing enzymes: these enzymes include not only ion-motive ATPases but also proteins involved in oxidative and photosynthetic reactions. Reconstitution has made an especially important contribution to the studies dealing with the mechanisms of transport, the determination of nature of the transported ions, the electrical properties of the transport mechanism, the ion/substrate stoichiometric ratios and the analysis of the coupling between transport systems. However, one should bear in mind that the concepts are not restricted to transport proteins, since reconstitution allows analysis of other important general properties of membrane proteins such as lipid–protein and protein–protein interactions, the topological features of proteins in the membrane, the specific roles of different subunits or ligand recognition and binding. Although it is difficult to list all the information published since the pioneering work of Racker and coworkers which started 20 years ago [1–5], a number of reviews are available concerning specific classes of membrane proteins such as receptors, substrate carriers, energy-conserving enzymes involved in oxidative phosphorylation, and ion-motive ATPases [6–17].

However, despite the extensive use and diverse applications of proteoliposomes, it must be stressed that the mechanism of their formation is in many ways surprisingly ill defined. This is to some extent understandable, as most of the researchers concerned were mainly interested in developing reconstitution methods that work for 'their' proteins and not in the physicochemical parameters involved in the reconstitution procedures. Consequently, the most important lesson to be learned from past reconstitution studies is that no one reconstitution procedure is likely to serve equally well for all membrane proteins, and that the experimental approach must be as broad as possible. Nevertheless, in the last few years, significant progress has been made in acquiring knowledge about the mechanisms of liposome formation as well as in understanding the physical behavior of detergent-lipid systems [18–21]. Due to this progress, it has been possible to develop a set of basic principles that limits the number of experimental variables and therefore the empirical approach to reconstitution experiments. In this connection, we developed, in our laboratory, a broad systematic method of assessing the efficiency of the reconstitution of different classes of energy-transducing membrane proteins, including bacteriorhodopsin, Ca^{2+}-ATPases and F_0F_1-type H^+-ATPases [22–30]. To this end we devised a new experimental strategy that allows greater insight into the mechanisms that trigger protein insertion into liposomes during the most commonly used procedure, namely detergent-mediated reconstitution. It is the purpose of this article to review, after a brief introduction describing the methods of preparing proteoliposomes, our personal experience in the reconstitution field. One of the main aims of the present review is to discuss and analyze our contribution to knowledge about membrane-reconstitution and it has been written in the hope that it will promote an integrated approach to this field, which has often seemed more like an art than a science. Besides the mechanisms of membrane protein reconstitution, the respective efficiencies and relevant advantages of the proteoliposomes reconstituted by this strategy will be considered and discussed in relation to both functional and structural studies of energy-transducing membrane proteins in model systems.

2. Strategies for membrane protein reconstitution into liposomes

From the analysis of the abundant literature concerning the reinsertion of membrane proteins into liposomes, four main technical strategies can be outlined (see Fig. 1), derived mainly from the strategies used to prepare pure phospholipid vesicles [31].

2.1. Organic solvent-mediated reconstitutions

Organic solvents have been widely used to prepare liposomes with large internal aqueous space and high capture efficiency in procedures including ethanol injection [32], ether infusion [33] and reverse-phase evaporation [34]. However, the usefulness of such strategies for the study of membrane proteins has been limited because they require exposure to organic solvents which often denature amphiphilic membrane proteins.

One manoeuvre used to reassemble membrane proteins into large liposomes has been reported by Darszon et al. [35]: large proteoliposomes could be formed by evaporating a solution of protein-lipid complex in apolar solvents followed by a rehydration with aqueous buffer. Most of the predominant population of multilamellar structures and amorphous material were then eliminated by sedimentation through sucrose, leading to giant proteoliposomes (5–300 μm) containing the protein in an active form. This technique has been applied to different classes of membrane proteins which include squid and bovine rhodopsins, cytochrome *c* oxidase, reaction center and acetylcholine receptor extracted into ether or hexane in the presence of soybean phospholipids. However, the inhomogeneity of the size distribution, the large proportion of multilamellar structures and the osmotic fragility of such proteo-

liposomes precluded detailed functional studies of the reconstituted proteins. A particularly attractive feature of this procedure might have been to enable the electrical properties of ions transport proteins to be studied with microelectrodes or patch-clamp techniques. However, for this purpose, other techniques which avoid the use of harmful organic solvents are preferred for the preparation of giant proteoliposomes [36,37].

The only suitable method reported to date for organic solvent-mediated reconstitution of membrane proteins is the reverse-phase evaporation method originally described by Szoka and Papahadjopoulos [34]. This strategy has allowed efficient incorporation of rhodopsin [38] and bacteriorhodopsin [39]. Large unilamellar proteoliposomes (0.2 to 5 μm) are formed from water-in-oil emulsion of phospholipid-protein-aqueous buffer in an excess of organic solvent, followed by removal of the organic phase under reduced pressure. Pentane, hexane, diisopropyl ether and diethyl ether have been commonly used as organic solvents. Bacteriorhodopsin proteoliposomes reconstituted by this method have been characterized in detail in our laboratory and were shown to meet all the following criteria of usefulness as model systems:

– Homogeneous size distribution : the heterogeneous size distribution of the crude preparation can be reduced by sequential extrusion through polycarbonate membranes thus permitting an homogeneous preparation of unilamellar proteoliposomes with a mean diameter centered around 0.2 μm [39,40].

– Homogeneous protein distribution among the liposomes [39,40].

– Good unidirectional orientation of the protein in the membrane (80–85% inside-out). Further detailed studies indicated that inside-out and right side-in oriented proteins were at least partially distributed in two different proteoliposomes populations [41]. These two functional subclasses of proteoliposomes could be separated using Sephacryl S-1000 gel-filtration chromatography leading to the largest proteoliposomes with 100% inwardly pumping activities [42].

– Efficient protein reconstitution in a large range of lipid-to-protein ratios (from 320 to 1 (w/w), i.e., from 25 to 8000 bacteriorhodopsin molecules per liposome) [40].

– Low H^+ and counterion permeabilities : proton/hydroxyl and K^+ permeability coefficients are of the order of $5 \cdot 10^{-5}$ cm s^{-1} [43,44].

– Very efficient biological activity : light-induced pH gradients as large as 2.5 pH unit can be generated across the membranes of these proteoliposomes [39,44].

Unfortunately, the lack of general procedures for the transfer into apolar solvents of other more hydrophilic membrane proteins in an active form has precluded the general use of this method, which should, in any case, be assessed with very hydrophobic proteins.

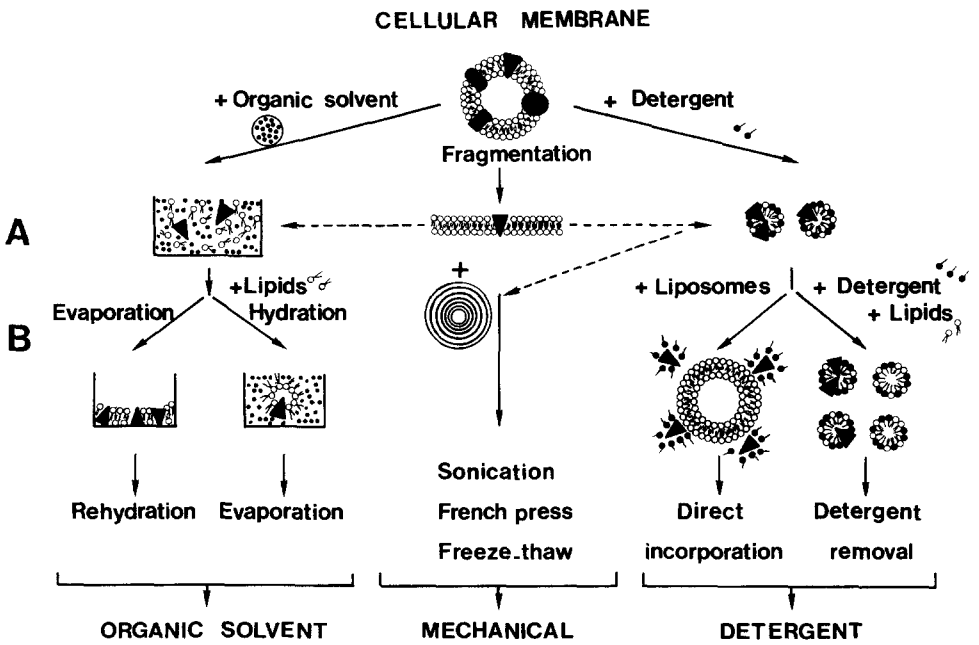

Fig. 1. Schematic representation of different strategies for the functional reassembly of membrane proteins into liposomes. Starting from purified cellular membranes of eukaryotic or procaryotic cells, membrane proteins can be isolated either in a mixed micelle with detergent, dissolved in an organic solvent or in an aqueous environment as aggregates or membrane fragments (frame A). Once isolated and purified, these membrane proteins are supplemented with an excess of phospholipids and can be reconstituted into liposomes following three main strategies (frame B): organic solvent-mediated reconstitutions (reverse-phase evaporation, rehydration of lipid-protein films); mechanical means (sonication, French press, freeze-thaw); detergent-mediated reconstitutions (detergent removal, dilution or direct incorporation).

2.2. Mechanical means

Putative mechanisms of vesicle formation by mechanical means have been reviewed elsewhere [19]. When dry phospholipid films swell in excess aqueous buffer, multilamellar lipid vesicles (MLV's) form spontaneously. To produce large unilamellar (LUV's) or small unilamellar vesicles (SUV's) which have higher free energies, some energy must be dissipated into the multilamellar system. The most widely used technique to prepare SUV's involves the sonication of MLV's, but this can be also performed by forcing a MLV's suspension through a French press [45]. The reverse transformation of SUV's into LUV's, MLV's or hydrated phospholipid aggregates by freeze-thawing [46] or dehydration-rehydration can also be envisaged from this kind of energetic consideration.

Sonication of a mixed suspension of lipids and isolated proteins has been widely used in the earlier stages of membrane protein reconstitution to demonstrate the function of these purified proteins for which detergent dialysis was inefficient [5]. If advantages of the sonication procedure are that it is rapid and requires no detergent, major

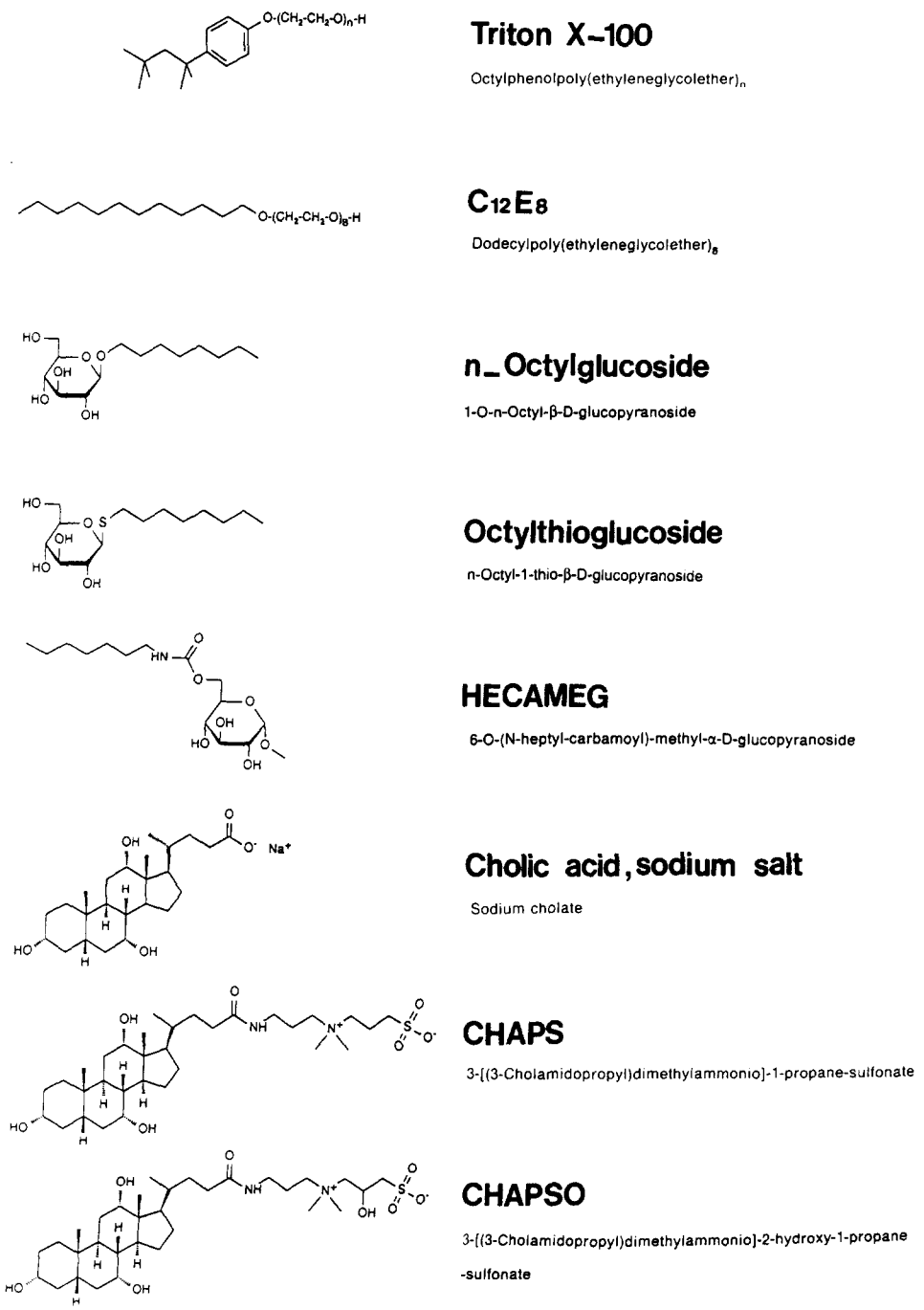

Fig. 2. Structural formula and conventional names of detergents useful for solubilization and reconstitution of membrane proteins.

crucial drawbacks can be irreproducibility, inactivation of many proteins by long sonication and small size of the resulting proteoliposomes (20–40 nm).

A great improvement was the freeze-thaw sonication procedure originally described by Kasahara and Hinkle [47] for the reconstitution of the D-glucose carrier from red blood cells. In this method, preformed sonicated liposomes are mixed with the protein and rapidly frozen in dry ice or liquid nitrogen. Upon thawing at room temperature, very large permeant proteoliposomes are formed which can be broken by a short sonication leading to a preparation of unilamellar functional proteoliposomes with diameters ranging from 20 to 200 nm. It has been suggested [46] that, during the rapid freezing, water molecules crystallize on the charged phospholipid interface, forming two frozen planes separated by the hydrophobic core of the membrane. The lipid bilayer is easily fractured and the exposed hydrophobic cores fuse to form large liposomes during the slow thawing. In this context high ionic force, sucrose or Ca^{2+} inhibit the fusion process which on the other hand requires negatively charged phospholipids. The freeze-thaw sonication strategy has been widely used because it is rapid and can be applied to proteins that are sensitive to sonication or to detergents. However, very few systematic studies of the orientation of the proteins after reconstitution have been performed, which make further interpretation and generalization of this procedure difficult. Additional disadvantages are the relatively wide range of sizes of the resulting proteoliposomes and, more important, the possibility of unfavorable perturbation of membrane protein structure (e.g., aggregation) due to subtle changes produced by the freeze-thaw process. At present, this process, combined with dehydration-rehydration cycles, is used mainly to produce giant proteoliposomes adapted to electrophysiological techniques [36,37].

2.3. Direct incorporation into preformed liposomes

In early variations of this approach, spontaneous incorporation of membrane proteins (cytochrome *c* oxidase, cytochrome b_5, F_0F_1-ATPase) into preformed sonicated liposomes was shown to be catalyzed by low cholate, lysolecithin or octyl glucoside concentrations [48–57]. Membrane proteins have also been incorporated in the absence of added detergents, but in this case the liposomes had to be of well-defined composition with a strict requirement for acidic phospholipids [51]. Importantly, protein incorporation was demonstrated to occur preferentially into liposomes of small diameter (20 nm) [8,51,53]. The similarities between the parameters involved in direct incorporation and those involved in membrane fusion led the authors to envisage direct insertion as the fusion of the lipid envelope of adhering protein with liposomes [8].

The mechanisms of direct incorporation were further analyzed in detail by Zakim and co-workers [14] using different classes of integral membrane proteins (cytochrome b_5, bacteriorhodopsin, cytochrome oxidase). Such incorporations which were inefficient into large liquid crystalline liposomes, required SUV doped with amphipatic contaminants such as cholesterol, short-chain phosphatidylcholines, detergents, lyso-derivatives or fatty acids. Two events appeared to be involved in forming the phospholipid-protein complexes: the first was the fast insertion of all proteins into a small percentage of total liposomes. The second event was slower, and consisted in a continuous fusion of proteoliposomes with the remaining SUV's. The features that promoted direct protein incorporation were described by the authors as 'defects' which could arise from transbilayer asymmetry, lateral immiscibility, thermal motion or geometrical constraints.

The main advantage of this strategy is that in all cases where it has been checked, the protein was found to be oriented unidirectionally in the membranes of the proteoliposomes (for reviews see [8,14]) (see section 5.2). However, important short comings of the method are that proteoliposomes have a wide size range and that protein is heterogeneously distributed among the liposomes due to inhomogeneous and/or incomplete fusion. Furthermore, the presence of the 'impurities' needed for efficient reconstitution can be a problem in the case of energy-transducing membrane proteins as it might increase the basic permeability of the resulting proteoliposomes.

2.4. Detergent-mediated reconstitutions

The most successful and frequently used strategy for proteoliposome preparation is that involving the use of detergents, because most membrane proteins are isolated and purified in the presence of detergents (Fig. 2, [58]). In the standard procedure, these proteins are first cosolubilized with phospholipids in the appropriate detergent in order to form an isotropic solution of lipid-protein-detergent and lipid-detergent micelles. Next, the detergent is removed resulting in the progressive formation of bilayer vesicles with incorporated protein.

There are various methods of detergent removal based on the following physicochemical properties of detergents: critical micelle concentration (cmc), which is defined as the concentration at which the detergent monomers are forming micellar aggregates, micellar size in relation to the aggregation number of detergents in a micelle and the hydrophilic-lipophilic balance (HLB) which relates to the amphiphilicity of the detergent (Fig. 3).

Detergents with high cmc's (cholate, deoxycholate, octyl glucoside, Chaps, Chapso) are easily removed by dialysis [59]. Using a flow-through dialysis cell [60] can be advantageous, as the rate of detergent removal can be carefully controlled and dialysis time largely decreased. Also with the addition of detergent-adsorbing beads outside the dialysis bags, the number of changes of buffer generally required during dialysis can be reduced [61]. Detergents with high cmc's generally form small micelles and can be

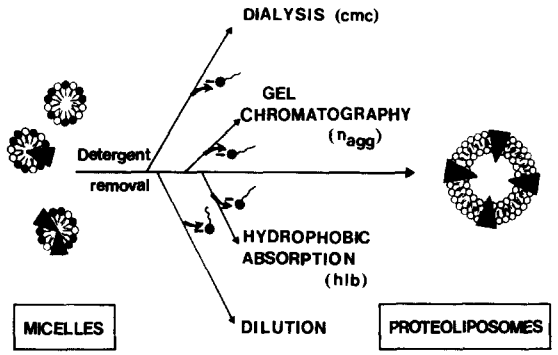

Fig. 3. Schematic representation of different strategies for detergent-mediated reconstitutions of membrane proteins. Purified membrane proteins (▲) are first co-solubilized in an excess of detergents (●) and phospholipids (○) to form an isotropic solution of binary and ternary micelles. Next the detergent concentration is lowered either by dilution or by removal of detergent by dialysis, gel chromatography or hydrophobic adsorption on polystyrene beads. The different methods for detergent removal are generally related to the physico-chemical properties of detergents: critical micelle concentration (cmc), micellar size (n_{agg}) and hydrophilic-lipophilic balance (hlb).

removed by gel-filtration techniques. In this case, the reconstitution micellar mixture is eluted through a gel-exclusion column: as the mixtures passe through the resin, the protein is integrated into liposomes which separate and elute from the column before the detergent. Depending upon the size of the micelles, one can use different gel-sized columns ranging from Sephadex G25 to Sephadex G200 [59]. The most significant advantage of this technique is its rapidity (avoiding long periods of contact between detergents and proteins) which, however, turns into disadvantages in terms of incomplete protein incorporation and also in terms of broader size distribution of proteoliposomes than found by dialysis [62].

Detergents which have a low cmc and consequently form large micelles are not readily removed by gel chromatography, and even less by dialysis, but can be efficiently removed through adsorption on hydrophobic resins (SM_2 Bio-Beads or Amberlite XAD) [63,64]. Such detergents include Triton X-100 and poly(oxyethylene glycol) detergents, but it should be stressed that the hydrophobic adsorption has been shown to be efficient for all kinds of detergents, including those with a high cmc (see section 5.3).

Another procedure for obtaining proteoliposomes from lipid-detergent-micellar solutions consists of diluting the reconstitution mixture [65]. Dilution lowers the detergent concentration to below its cmc and proteoliposomes form spontaneously. Detergents with high cmc's such as cholate or octyl glucoside have been used and generally the dilution was followed by centrifugation of the diluted proteoliposomes. Although employed with some success for reconstitution of different classes of proteins, the dilution technique has failed in many cases. However, this could be related to two crucial parameters not systematically explored: (i) leakiness of the proteoliposomes (the residual detergent should in any cases be removed by previously described procedures); (ii) rate of dilution, which has been shown to affect drastically the homogeneity of protein distribution and the morphology of the resulting proteoliposomes (unpublished results on Ca^{2+}-ATPase reconstituted by cholate dilution; see also [66–68]).

From the abundant literature, it appears that reconstitution from lipid-protein-detergent mixtures yield proteoliposomes of different sizes and compositions, depending

Table 1
Criteria for ideal reconstituted proteoliposomes

Criteria	Structural and functional advantages	Techniques
Large size of proteoliposomes	Lipid packing	Electron microscopy
	Low lipid-to-protein ratios	Gel chromatography
	Intraliposomal bulk water	Light-scattering
	High solute or ion accumulation	Internal volume
Uniformly sized proteoliposomes	Kinetic studies transport	Electron microscopy
		Gel chromatography
Uniform lipid-to-protein ratios	Lipid–protein interactions	Sucrose gradient
	Protein–protein interactions	Freeze-fracture electron microscopy
	Kinetic studies of transport	
	Estimation of efficient internal volume	
Asymmetric protein orientation	Protein-protein interactions	Proteolysis
	Topology, topography	One-sided inhibitors
	Kinetic studies of transport	Activities ± detergents
	Stoichiometries (substrate-transport species)	
Low passive permeability	High solute accumulation	pH jumps with entrapped fluorescent probes
	pH gradient	
	Transmembrane electrical potentials	Permeability measurements
	Countertransports	Release of encapsulated probes
	Probe encapsulation	

on the nature of the detergent, the particular procedure used to remove it, the protein and lipid composition, the ionic conditions and also the precise conditions of initial detergent solubilization. Therefore, as stated in the Introduction, it is not surprising that each membrane protein responds differently to the various reconstitutions procedures, and for a long time the approach to these procedures has been entirely empirical. This empirism is even more striking when one considers the ideal criteria which have to be fulfilled for optimization of the potential use of proteoliposomes in membrane protein research. Besides the need for conditions that preserve the integrity and activity of the protein under study, the following important criteria should be considered: the morphology and size of the proteoliposomes, the homogeneity of their size and protein distribution, the number of protein units incorporated, the final orientation of the incorporated transmembrane protein and the permeability of the proteoliposomes.

All the essential requirements for an efficient and serious reconstitution are listed in Table 1, together with the techniques generally used to check them. At this point it is important to stress the necessity of such thorough characterization of the reconstituted systems, in addition to the necessary functional assays. This is the only way to achieve optimal reconstitution and avoid artifactual interpretation of the results. Furthermore, it is a key factor for obtaining detailed information on the mechanisms involved in membrane protein reconstitution.

3. Proteoliposome formation during detergent-mediated reconstitutions

The molecular mechanisms for the formation of proteoliposomes upon detergent removal from a detergent/lipid/protein mixture are only partly known. The simplest scheme relies on the simplest idea that reconstitution (i.e., the process by which micelles form vesicles) is the mirror image of solubilization by detergent addition (i.e., the process by which vesicles form micelles), involving probably the same sequence of intermediate structures [58,69].

3.1. Liposome solubilization

The 'three-stage' model

The process of liposome solubilization by different detergents has been the subject of considerable investigation. The methods used include turbidimetry [22,24,70,71], quasielastic light scattering [66,72], fluorescence energy transfer [73,74], magnetic resonance spectroscopy [22,27,71,75,76] centrifugation experiments [27,71], and electron microscopy [77,78]. The results of most of these studies have been related to a 'three-stage model' describing the interaction of detergents with lipidic bilayers (for reviews see [18,20,21] : for a given concentration of liposomes, three stages in the solubilization process are

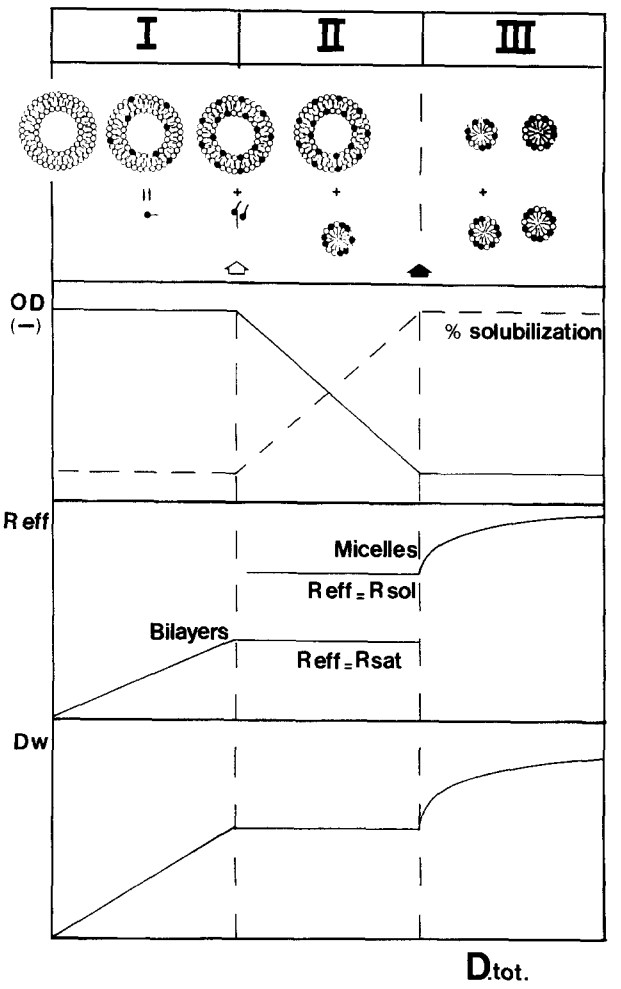

Fig. 4. Idealized 'three-stage' profile of the solubilization process of liposomes by increasing concentration of detergents. The idealized phase diagram is adapted from results in Refs. [22,24]. First panel: diagram of the solubilization process of liposomes. The regions designated by I, II and III correspond respectively to ranges of detergent concentration in which liposomes only, liposomes and micelles or micelles only are present. White and black arrows correspond to the onset and total solubilization, respectively. Second panel: changes in turbidity of the lipid-detergent suspension and percent of lipid incorporated into mixed micelles. Third panel: mole fractions of detergent in bilayers and/or in micelles. Fourth panel: concentration of monomeric detergent. In stage designated by I, an increase in the total detergent concentration increases both the concentration of monomeric detergent and the mole fraction of detergent in the liposomes but no micelles are formed. During this stage, turbidity is slighltly affected. In stage designated by II, liposome solubilization occurs with the appearance of coexisting population of mixed micelles leading to a large decrease in turbidity. In this stage, the mole fraction of detergent in the liposomes plateaus at its saturation level (R_{sat}), while the micelles contains a different, but constant, mole fraction of detergent (R_{sol}). In stage designated by III, the lipids are totally solubilized and only mixed lipid-detergent micelles exist.

apparent, depending on the nature and concentration of the detergent (Fig. 4).

In stage I, raising the total detergent concentration raises both the concentration of monomeric detergent in the aqueous phase and the mole fraction of detergent in the

bilayer. Detergent incorporation into the bilayer phase can be described with a well-defined partition coefficient. Although our studies with $C_{12}E_8$ [24] and Triton X-100 (unpublished results) have shown an ideal mixing of lipids and detergents throughout this stage, data acquired with other detergents indicate that a true detergent equilibrium partition is only valid at low mole fractions of bilayer-incorporated detergent [79]. In this connection, Bayerl et al. [80] suggested two interactions sites of detergents in liposomes, one at the interface region and the other in the hydrocarbon chain region of the liposomes: the partition of the detergents between these two sites depended upon the intrinsic lateral pressure of the bilayer. Thus, obviously, the partition behavior of detergents into the bilayer will depend upon the nature of the phospholipids, the size of the liposomes, the nature of the detergent and probably the method of measurement.

It is noteworthy that, while bilayer solubilization does not occur in this stage, the detergents induce structural perturbations, as shown by the large increase in membrane permeability [22,24,73], and in some cases by the changes in the size and morphology of the vesicles. In particular, small sonicated vesicles exhibit a complex behavior in the presence of sub-solubilizing detergent concentrations: the detergents act as 'wedges', disrupting the vesicle structure and thus releasing it from the lateral strain imposed by the high curvature of these small liposomes. These very unstable open vesicles display a natural tendency to aggregate and/or fuse [81–83].

Stage I ends up when the bilayer becomes 'saturated' with detergent, the aqueous detergent concentration being approximately equal to the cmc.

During stage II, the system undergoes a structural transition from lamellar structures (i.e., detergent-saturated liposomes) to a population of phospholipid-detergent micelles. The transition is not infinitely sharp and there is a region where both of these mixed amphiphilic structures coexist. We determined quantitatively [24] that the effective detergent-to-phospholipid ratios in the vesicles and in the mixed micelles remained constant throughout most of stage II and respectively corresponded to the critical detergent-to-phospholipid ratios at which stage II began (R_{sat}) and finished (R_{sol}). Throughout the entire bilayer to micelle transition, detergent-saturated vesicles and lipid-saturated micelles coexisted, and only their relative proportion varied with increasing detergent concentration.

In stage III, the phospholipids are completely solubilized into mixed micelles, leading to optically transparent solutions. As the total detergent concentration increases, the mole fraction of detergent in the micelles increases, with a concomitant decrease in the size of these micelles [22,24,71,73,74].

Parameters describing the solubilization process

For any given detergent/lipid mixture, the most important factor in determining the type of aggregates (mixed vesicles or mixed micelles) is the detergent to lipid molar ratio in the aggregates, herein denoted the effective ratio R_{eff}. Since the concentration of detergent in detergent-lipid mixed aggregates (vesicles or micelles) is equal to the difference between the total detergent concentration (D_{total}) and its aqueous monomeric concentration (D_{water}), R_{eff}, has been defined as shown in Eq. (1):

$$R_{eff} = D_{total} - D_{water}/[Lip] \quad (1)$$

Thus, the process of solubilization is generally described by Eq. (2) [20–22,24]:

$$D_{total} = D_{water} + R_{eff}.[Lip] \quad (2)$$

where [Lip] and D_{total} are the total lipid and detergent concentrations, D_{water} is the monomeric detergent concentration and R_{eff}, the effective detergent-to-phospholipid ratio in mixed aggregates. R_{eff} will be referred as R_{sat} at the onset of solubilization and will correspond to the detergent to lipid ratio in detergent-saturated vesicles. R_{eff} will be referred as R_{sol} at total solubilization and will correspond to the detergent to lipid ratio in mixed lipid-detergent micelles.

According to Eq. (2), it may be possible to calculate the critical effective detergent to phospholipid ratio at which the lamellar to micellar transition begins and finishes from the linear dependence of the critical detergent concentration at which phase transformation occurs (D_{total} for the onset and total solubilization) over the lipid concentration (Lip). The values of the slopes of the solid lines schematically represented in Fig. 5 are that of R_{sat} and R_{sol}, while the extrapolated intercepts equal the aqueous monomeric detergent concentration.

Table 2 summarizes the results obtained during the course of our studies on the solubilization process by different detergent of liposomes prepared by reverse-phase evaporation.

On a more technical standpoint, one important general conclusion of our studies [22,24] (see also [21]) was that the 'three-stage' model could be easily visualized and analyzed in many cases through changes in turbidity of the lipid-detergent suspensions (2nd panel in Fig. 4). In turbidity versus detergent concentration curves, the turbidity is initially only slightly affected during stage I. Further detergent addition results in a large decrease of turbidity corresponding to the gradual solubilization of vesicles during stage II. Stage III is characterized by a complete solubilization of lipids into mixed micelles and the solutions become optically transparent. Perfect agreements between turbidity measurements and NMR or centrifugation studies, demonstrated that the point at which turbidity started to decrease markedly could be referred to the onset of solubilization, while the point at which optically transparent solutions were obtained could be referred to the total solubilization. However, at this point, we would like to stress that such ideal turbidimetric behavior was not always observed and with some detergents (Chaps, Chapso and glycosylated

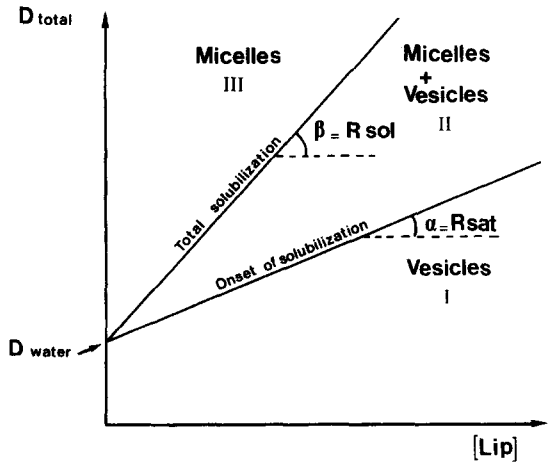

Fig. 5. Analysis of the solubilization process from the dependence of the total detergent concentration for onset and total solubilization upon the lipid concentration. When the critical detergent concentrations at which the vesicular to lamellar transformation begins (onset of solubilization) and finishes (total solubilization) are plotted as a function of total lipid concentration, straight lines are obtained (see Refs. [21–24]). The two lines of constant slope α and β can be described by equation:
$D_{total} = D_{water} + \alpha.[Lip]$ and $D_{total} = D_{water} + \beta.[Lip]$
Since $D_{total} = D_{water} + D_{bilayer}$ and defining $R_{eff} = D_{bilayer}/[Lip]$, the process of solubilization can be described by the general equation:
$D_{total} = D_{water} + R_{eff}[Lip]$
with $R_{eff} = R_{sat} = \alpha$ at the onset of solubilization. and $R_{eff} = R_{sol} = \beta$ at total solubilization.
Extrapolation of the two lines to zero lipid concentration defines the concentration of the monomeric detergent (D_{water}) in equilibrium with either the detergent-saturated liposomes or the mixed lipid-detergent micelles. I, II, III correspond to the three stages of the solubilization process described in Fig. 4.

derivatives) important structural reorganization have been observed during stage II, rendering difficult an accurate analysis of the solubilization process by turbidimetry. Such observations together with the fact that changes in turbidity may be caused by many factors other than liposome-micelle transitions (e.g., changes in the refractive index of water or membranes, changes in liposome or micelle size, aggregation) point out for the need of documenting, in any cases, the solubilization process with other techniques. Furthermore, this technique does not bring any information about the morphology of the intermediates between vesicles and small spheroidal mixed micelles which is actually more open to discussion ([77,78], see Section 3.2).

3.2. Liposome formation during detergent removal from detergent-lipid micelles

In the simplest scheme, the gradual removal of detergent from a micellar lipid-detergent solution should lead to reassociation of lipids into closed bilayer in a sequence that is the mirror image of the solubilization process [20,58,69]. Although the information about this is scarce, it has been shown to occur in practice. For example, the process of liposome reconstitution upon detergent removal from mixed phospholipid-$C_{12}E_8$ micelles has been analyzed in detail in our laboratory [24]. Our most interesting finding was that, during detergent removal, liposome formation took place in three distinct stages, which were the symmetrical opposites of those observed during the solubilization process. Furthermore, the striking similarities between the critical detergent-to-phospholipid ratios at which phase transformations occurred, as well as the composition of the lamellar and micellar structures present in solution during the reconstitution and solubilization processes demonstrated in addition that these processes were quantitatively symmetrical. Similar conclusions could be drawn from our studies on Triton X-100-mediated reconstitutions (unpublished results). However, it should be noted that our results regarding the quantitative description of the intermediate stages during liposome formation do not preclude any information concerning the size and shape of the different intermediates formed, which can be very different in solubilization and in reconstitution experiments. In this context a model has been proposed by Lasic [19] which in addition to the micelle-vesicle transition comprises two other aspects such as a micellar equilibration and a post-vesiculation size transformation. The basic concepts for micellar equilibration are that as the detergent molecules are removed from mixed micelles, a series of micelle-micelle interactions is initiated, to minimize the unfavorable energy resulting from the consequent exposure of lipid hydrophobic regions to the aqueous medium. This results in large oblate mixed micelles growing up to a critical size and composition that is sufficient to cause bilayer closure and thus vesicle formation. However, this model of micellar equilibration before the onset of liposome formation is not absolutely clear due to the inherent

Table 2
Parameters describing the solubilization process of liposomes by different detergents

Detergent	R_{sat} (mol/mol)	R_{sol} (mol/mol)	D_{water} (mM)
Triton X-100	0.64	2.5	0.18
Sodium cholate	0.3	0.9	2.18
$C_{12}E_8$	0.6	2.1	0.2
Octyl glucoside	1.3	2.6	17
Octyl thioglucoside	2.8	5.8	7
Hecameg	n.d.	4.0	17.5
Chapso	0.38	0.73	1.85–2.4
Chaps	0.4	1.04	2.8 –3.5

Liposomes were prepared by reverse-phase evaporation (egg phosphatidylcholine:egg phosphatidic acid, 9:1 mol/mol) and solubilization by different detergents was analyzed using turbidimetry, ^{31}P-NMR and centrifugation experiments (Refs. [22–24] and unpublished results). As detailed in the text and legends of Fig. 5:
R_{sat} = effective detergent to phospholipid molar ratio in detergent-saturated liposomes.
R_{sol} = effective detergent to phospholipid molar ratio in mixed micelles.
D_{water} = aqueous monomeric detergent concentration in equilibrium with either detergent-saturated liposomes or mixed micelles.

difficulty of analyzing the instable intermediate structures throughout detergent removal. In this connection, slightly different models have been proposed, involving the formation of intermediates, such as large bilayered aggregates [84], extended rod-like structures [77] or flexible cylindrical micelles [78]. A second key step in liposome reconstitution relies on a post-vesicular size transformation. Indeed the initially formed vesicles continue to undergo a size transformation process, as long as the level of residual detergent remains high. This size transformation has been suggested to occur through detergent-promoted intervesicular mass-transfer [66,70,85] and to depend on the experimental conditions they encounter before they attain their final stable state. For example, Almog et al. [66,70] reported that the size and homogeneity of lipid vesicles obtained by dilution of micellar cholate-lipid solutions depended upon the rate and extent of dilution: rapid dilution to very low cholate concentration produces a stable population of small vesicles while less extensive dilution leads to the formation of vesicles that subsequently grow in size.

In conclusion, despite disagreements in the structure and the size of the different intermediates between pure micelles and pure liposomes, many of the results reported in the literature are consistent with the simplest proposed scheme for liposome formation upon detergent removal and which comprises three stages: micellar equilibration up to the onset of liposome formation (corresponding to stage III in the solubilization scheme), progressive transformation of all micelles into detergent-saturated liposomes (corresponding to stage II in the solubilization scheme), and detergent removal from detergent-saturated liposomes (corresponding to stage I in the solubilization scheme).

3.3. Proteoliposome formation during detergent removal

A main characteristic of a lipid-protein-detergent micellar solution is its inhomogeneity with regard to size and composition of micelles: typically binary lipid-detergent and ternary lipid-detergent-protein micelles coexist. This heterogeneity complicates the simple scheme of detergent-mediated reconstitution of pure liposomes reported above. Indeed, a key step in proteoliposome reconstitution experiments is the insertion of the protein into the bilayer of the liposomes formed. With regard to protein-lipid association during detergent removal from mixed binary and ternary micelles, two mechanisms were proposed by Eytan [8] based on the results of reconstitution of different membrane proteins, using different techniques and different detergents: (1) the protein simply participates in the membrane formation process, which corresponds to the micellar-lamellar phase transformation; or (2) liposomes are first formed by partial detergent removal, and only after further removal is the protein inserted into the preformed detergent-doped liposomes.

Since the formation of lipid-protein complexes from a micellar solution depends on the changes in local composition which occur inside the particles formed upon detergent removal, then the aqueous solubility of each constituent and the composition of the initial mixed micelles can be key factors in determining the products of reconstitution. Furthermore, Eytan [8] suggested that the rate of detergent removal might be crucial in proteoliposome formation. He proposed that upon slow detergent removal, vesicle formation might precede protein incorporation into the lipid bilayer, while upon rapid detergent removal, protein incorporation occurred during vesicle formation which corresponded to the micellar-lamellar transition. In this framework, the rate of detergent removal was shown to influence drastically the homogeneity of protein distribution during octyl-glucoside-mediated reconstitution of rhodopsin [67,68]: rapid detergent removal caused simultaneous coalescence of lipid-detergent and rhodopsin-lipid-detergent micelles, leading to a homogeneous end-product, while gradual detergent removal allowed protein-free and protein-containing micelles to assemble into vesicles at different steps, leading to a more heterogeneous population of proteoliposomes. In addition, Helenius at al. [69] demonstrated that a critical factor in determining the mechanism of protein reconstitution was the state of aggregation of the protein at the point when the liposomes began to form from the solubilized lipids. Other factors involved include micelle size, lipid composition and ionic conditions.

From all these considerations, it is obvious that we do not yet clearly understand the mechanisms of lipid-protein association during detergent-mediated reconstitutions, which makes it difficult to rationalize all the information reported and to establish basic principles that would limit the number of variables to be checked.

4. Mechanisms of lipid-protein association during detergent-mediated reconstitutions

4.1. A new strategy to study reconstitution processes

In a practical detergent-mediated reconstitution procedure, experimental monitoring of the mechanism by which proteins are inserted into liposomes is problematic because lipid-protein-detergent dispersions are heterogeneous populations of inherently instable binary and ternary micelles which may coalesce at different stages of detergent removal. Furthermore, one cannot reduce the detergent concentration slowly enough to follow the equilibration process and organization of the vesicles and micelles during detergent removal.

To allow realistic experimental monitoring of the mechanisms by which proteins may associate with lipids, we developed a new strategy based on the idea that reassociation of lipids and proteins upon selective removal of the

detergent from a detergent/lipid/protein mixture is the mirror image of the solubilization process. Accordingly, the standard procedure for studying the incorporation of membrane proteins was carried out in three distinct steps (Fig. 6):

1. Stepwise solubilization of preformed liposomes: liposomes prepared by reverse-phase evaporation were resuspended at the desired concentration and treated with different amounts of detergent throughout the entire range of lamellar to micellar transition as described in the above section.
2. Protein addition: after incubation sufficient for detergent equilibration, a solution of solubilized monomeric protein was added to give the desired final lipid-to-protein ratio. The detergent/protein/phospholipid mixtures at each accurately adjusted step of the lamellar to micellar transition were kept at room temperature for 1 min to 2 h under gentle stirring.
3. Detergent removal: this was generally performed by successive additions of SM_2-Biobeads. After detergent removal, the resulting proteoliposomes were analyzed for protein incorporation, protein orientation and biological activities.

Such systematic studies were performed using essentially three prototypic energy-transducing membrane proteins: bacteriorhodopsin from *Halobacterium salinarium* (M_r 27000; prototype of membrane proteins with seven transmembrane α-helices; mainly hydrophobic; for a review see [86]); Ca^{2+}-ATPase from sarcoplasmic reticulum (M_r 110000; prototype of type P-ATPase; 10 trans-

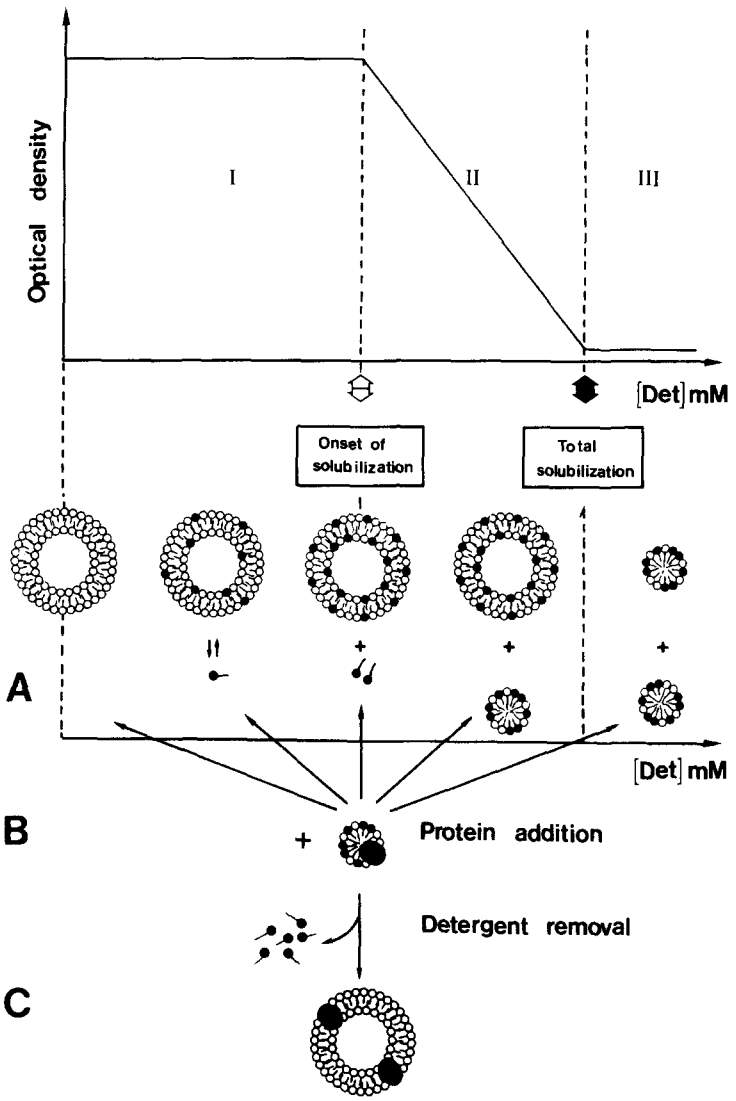

Fig. 6. Schematic representation of a new strategy for membrane protein reconstitution. The standard procedure for reconstituting membrane proteins was carried out in three different steps: (A) Stepwise solubilization of preformed liposomes (the solubization process can be qualitatively analyzed through turbidimetry). I, II, III correspond to the three stages of the solubilization process described in Fig. 4. White and black arrows correspond to the onset and total solubilization, respectively. (B) Protein addition at each step of the lamellar to micellar transition. (C) Detergent removal (by hydrophobic adsorption onto polystyrene beads).

membrane α-helices and a large hydrophilic part; for a review see [87]); H^+-ATPase from spinach chloroplasts (M_r 550000; prototype of F_0F_1-type H^+-ATPases; an F_0 hydrophobic part and a very large F_1 hydrophilic part; both F_0 and F_1 are composed of many subunits; for a review see [88]).

For each protein, the resulting vesicles were analyzed with respect to protein insertion and orientation in the membrane by freeze-fracture electron microscopy, sucrose density gradients, proteolytic digestions, one-sided inhibitors and biological activity measurements.

4.2. Mechanisms of lipid-protein association

From the results of our reconstitution studies [23,25,26,28] we were able to identify three mechanisms by which proteins can associate with phospholipids to give functional proteoliposomes (Fig. 7).

(1) The results of the reconstitution with sodium cholate demonstrated that proteoliposome formation only arose from detergent depletion of protein-lipid-detergent micelles. No protein incorporation into preformed liposomes could be detected, even when the liposomes were destabilized by saturating levels of cholate. Proteoliposome formation was linearly related to the percentage of lipid solubilization in the initial lipid-detergent suspension. Maximal activities and homogeneous protein distribution were measured in samples reconstituted from isotropic micellar solutions (Fig. 7A).

(2) In the case of Triton X-100-mediated reconstitutions (Fig. 7B), even though no protein was found to be associated with phospholipids until the starting material contained mixed micelles, the efficiency of the reconstitution was not related to the amount of mixed micelles initially present in the incubation medium. Optimal reconstitutions were detected in samples reconstituted from Triton X-100-phospholipid-protein suspensions, in which about 60–70% of the phospholipids were still present as Triton X-100-saturated liposomes. A time-dependent protein incorporation of about 1 h was observed, suggesting a transfer of the protein molecules initially present in the micelles to the detergent-saturated liposomes still present in the incubation medium. In the case of bacteriorhodopsin and/or H^+-ATPase, the transfer was total, leading to the

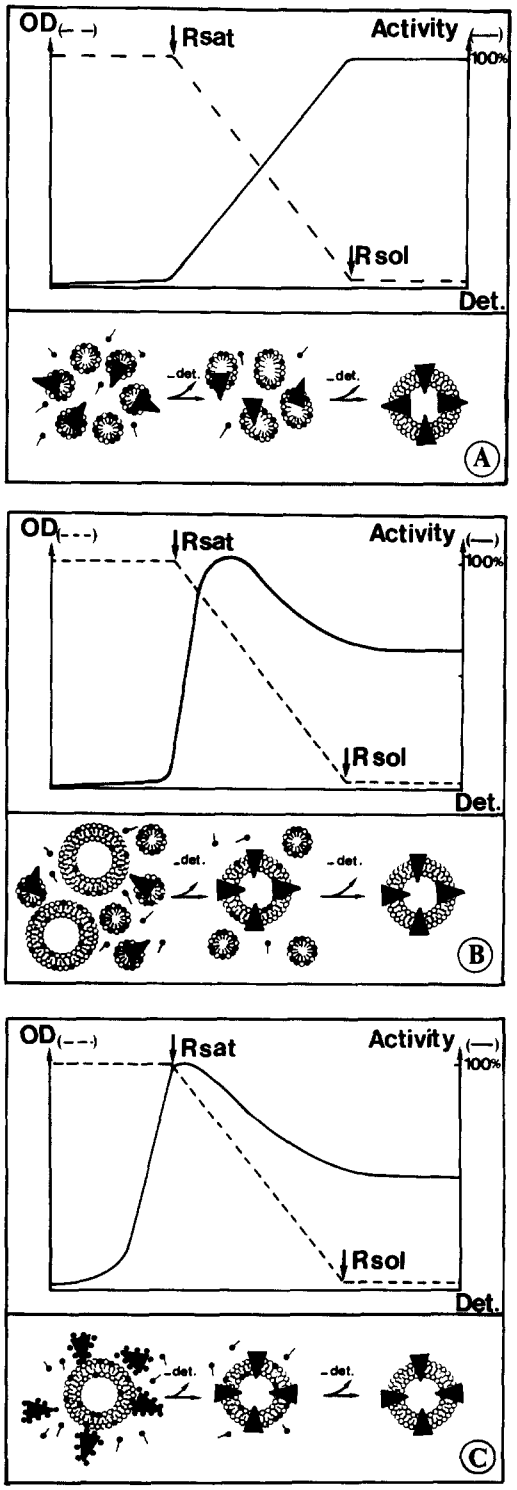

Fig. 7. Schematic representation of the different mechanisms of protein-lipid association during step-by-step detergent-mediated reconstitutions. Proteoliposomes were reconstituted according to the general strategy described in Fig. 6 using different detergents. The optical density of phospholipid-detergent-protein before detergent removal and the resulting biological activities of the proteoliposomes after detergent removal are plotted as a function of the initial total detergent concentration. The idealized representations are adapted from Refs. [22–28]. Panel A: cholate-mediated reconstitutions. Optimal proteoliposome formation arises from depletion of ternary mixed micelles and maximal activities are obtained when starting from totally solubilized protein/phospholipid mixtures. Panel B: Triton X-100-mediated reconstitutions. Optimal proteoliposome formation arises from protein transfer from mixed micelles to detergent-saturated liposomes and maximal activities are obtained when starting from protein-phospholipid mixtures where only about 25% of the liposomes are solubilized. Panel C: Octylglucoside-mediated reconstitutions. Optimal proteoliposome formation arises from direct incorporation of the protein into preformed detergent-saturated liposomes and maximal activities are obtained when protein is added to detergent-saturated liposomes. (Importantly, it should be noted that final protein orientation is unidirectional in panel C and random in panel A.)

formation of a homogeneous proteoliposome preparation with a final lipid-to-protein ratio similar to the initial ratio [23,26]. In the case of Ca^{2+}-ATPase, the transfer was partial, leading to the formation of heterogeneous proteoliposome preparation containing about 30% of protein-free liposomes [28].

(3) The results of octyl-glucoside-mediated reconstitutions (Fig. 7C) indicated that reconstitution was optimal when it started from a suspension at a detergent-to-phospholipid ratio around the critical ratio for the onset of liposome solubilization. This permitted the formation of proteoliposomes by direct incorporation of the protein into preformed liposomes, provided they were first destabilized by saturating levels of octyl glucoside. Such incorporation was observed to occur very rapidly, since after 2 min incubation of solubilized protein with octyl-glucoside-saturated liposomes, the resulting activities were already maximal. Furthermore, when two reconstitutions were performed in one of which the solubilized protein was added to octyl-glucoside-treated liposomes and in the other, the appropriate amount of detergent was first added to the protein and only then to the preformed pure liposomes, identical results were obtained after 2 min of incubation. Consequently, there must have been rapid equilibration; i.e., movement of protein and/or phospholipid between the micelles and untreated liposomes on the one hand, and the micelles and detergent-saturated liposomes on the other. Finally, density gradient centrifugation and electron microscopy also revealed that protein incorporation was complete and relatively homogeneous among the liposomes.

Table 3 summarizes the results from the different proteins studied, concerning the mode of optimal lipid-protein association, depending on the nature of the detergent. The first clear implication of these results is that the mode of protein-lipid association depends more on the nature of the detergent than on the structure of the protein. Interestingly, our findings on the different ways by which bacteriorhodopsin, Ca^{2+}-ATPase and H^+-ATPases associate with phospholipids in the presence of detergents may have some bearing on the observations of other authors. With regard to the ability of detergents to incorporate proteins into preformed liposomes, octyl glucoside proved, in many instances, useful in facilitating the direct incorporation of membrane protein into bilayer membranes [69,89,90]. On the other hand, detergent-mediated reconstitutions of various membrane proteins have been described to be more efficient when starting from partially solubilized material as well in the presence of Triton X-100 ([91]; anionic band 3 from erythrocyte membrane, Boulter, J., Taylor, A. and Watts A., personal communication) than in the presence of Chaps [92]. From all these considerations it is tempting to extend the mechanisms depicted in Fig. 7A–C to the reconstitution of most membrane proteins.

However, additional experiments to clarify the 'inconsistent' observation that the Triton X-100-mediated mechanism reported for bacteriorhodopsin and/or H^+-ATPase was inefficient for Ca^{2+}-ATPase demonstrated that another key factor in determining the final proteoliposome composition was the state of aggregation of the protein in the incubation medium. More precisely, centrifugation experiments indicated that on removal of a small amount of detergent from Triton X100-protein-lipid micelles, Ca^{2+}-ATPase molecules aggregated at the early stage of the vesiculation process, leading, after complete detergent removal, to heterogeneous preparations composed of aggregated protein-rich and protein-free liposomes [28]. Freeze fracture electron microscopy and transport data further demonstrated that the mechanisms of Ca^{2+}-ATPase reconstitution were chiefly driven by the tendency towards self-aggregation of this protein upon slight changes in the detergent concentration. The efficiency of a detergent was then related to its ability to transfer and disperse the aggregated Ca^{2+}-ATPase molecules among the protein-free liposome population.

Table 3
Steps in the lamellar-to-micellar transitions where optimal reconstitutions of different membrane proteins are observed

Protein	Detergent	Step of liposome solubilization		
		onset	intermediate	total
Bacteriorhodopsin (*Halobacterium salinarium*)	Octyl glucoside	+		
	Sodium cholate			+
	Triton X-100		+	
H^+-ATPase (Spinach chloroplast)	Octyl glucoside	+		
	Sodium cholate			+
	Triton X-100		+	
Ca^{2+}-ATPase (Sarcoplasmic reticulum)	Octyl glucoside	+		
	Sodium cholate			+
	Triton X-100			+
Bacteriorhodopsin + H^+-ATPase (Thermophilic bacillus PS3)	Octyl glucoside	+		
	Sodium cholate			+
	Triton X-100		+	
Bacteriorhodopsin + H^+-ATPase (Chloroplasts; mitochondria)	Octyl glucoside	+		
	Sodium cholate			+
	Triton X-100		+	

This table is adapted from results in Refs. [23–28].
Onset of liposome solubilization corresponds to detergent concentrations for which only detergent-saturated liposomes are present (end of stage I).
Intermediate liposome solubilization corresponds to detergent concentrations for which about 75% detergent-saturated liposomes coexist with 25% mixed micelles (stage II).
Total solubilization corresponds to detergent concentrations for which only mixed lipid-detergent micelles are present (stage III).

4.3. A model for proteoliposome formation from micellar solutions

Hypothetical model

From all our studies, we can now propose a general model for membrane protein reconstitution by detergent

removal from micellar solutions. Such a model includes the role of the nature of the detergent, of the rate of detergent removal and of the tendency for aggregation/oligomerisation of the protein. This model is schematically depicted in Fig. 8. Initially mixed lipid-detergent and lipid-protein-detergent micelles are present. Depending upon the tendency for self aggregation of the protein, two main schemes may occur.

(1) For membrane proteins such as Ca^{2+}-ATPase, i.e., with a large propensity for self-aggregation we propose two sequences of events related to the rate of detergent removal (Fig. 8, scheme A).

Upon slow detergent removal (process 2 in scheme A), we propose that as the detergent concentration is initially lowered, protein-rich structures are first formed. Then, as this concentration is further lowered, micelles containing lipids are disrupted, leading to the formation of detergent-saturated liposomes. At this stage we suggest that the dispersal of the protein among the liposomes depends on the ability of detergent to incorporate the 'aggregated' protein into the preformed liposomes. Our results indicate that such incorporation is negligible, partial and total in the presence of $C_{12}E_8$ (process 2c), Triton X-100 (process 2b) and octyl glucoside (process 2a), respectively. What exactly happens at the beginning of detergent removal is not clear, but could simply be related to one of two factors: (i) lipid-protein-detergent micelles are less stable than lipid-detergent micelles, so that the detergent is preferentially removed from the ternary micelles, resulting in the initial formation of protein-rich structures; (ii) the detergent is removed from both types of micelle, but protein molecules tend to aggregate upon destabilization of the ternary micelles. Although we have no definite proof, it is tempting to generalize this model to membrane proteins which tend to aggregate upon small variations in the detergent, protein and/or phospholipid contents.

Upon fast detergent removal (process 1 in scheme A), the disruption of both types of micelles is simultaneous, resulting in the mixing of their components. Whatever the nature of the detergent, the proteoliposomes formed under these conditions are a more accurate reflection of the overall composition of the starting solutions, and are thus more homogeneous.

Finally, for cholate-mediated reconstitution, we propose that proteoliposome formation only arises from micelle coalescence, whatever the rate of detergent removal (process 1 in scheme A).

(2) For other membrane proteins with a lower tendency for aggregation/oligomerisation or which give ternary micelles as stable as binary micelles, both types of micelle will disrupt simultaneously upon detergent removal and the protein will simply participate in the membrane formation process which corresponds to the phase transition from a micellar solution to a lamellar membrane phase

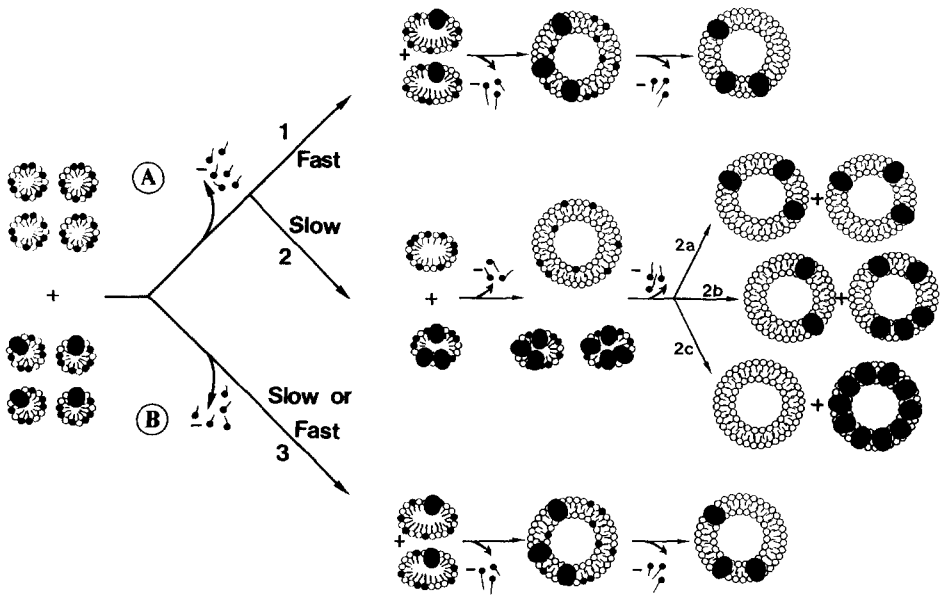

Fig. 8. Schematic representation of the mechanisms by which proteins can associate with phospholipids upon detergent removal from micellar detergent-lipid-protein solutions. The scheme suggests a possible sequence of events leading to proteoliposome formation upon detergent removal from a mixture of lipid-detergent and lipid-detergent-protein micelles. Part A of this scheme corresponds to the reconstitution of Ca^{++}-ATPase (i.e., proteins which tend to aggregate or which give unstable detergent-protein-phospholipid ternary micelles). Upon fast detergent removal, all the micelles coalesce, leading to homogeneous ternary micelles which reorganize in proteoliposomes upon further detergent removal (scheme 1). Upon slow detergent removal, detergent is not removed homogenously from ternary and binary micelles and a mixture of detergent-saturated liposomes and lipid-protein-detergent aggregates is formed. Depending upon its nature, the remaining detergent can catalyze total (scheme 2a), partial (scheme 2b) or no (scheme 2c) protein incorporation. Part B of this scheme corresponds to the reconstitution of bacteriorhodopsin (i.e., proteins which give ternary micelles as stable as binary micelles). Whatever the rate of detergent removal, both types of micelle initially present coalesce, leading to homogeneous micelles containing lipids and proteins which, upon further detergent removal, transform into proteoliposomes.

(Fig. 8, scheme B). Such a sequence of events would be valid for explaining formation of bacteriorhodopsin or H^+-ATPase proteoliposomes by detergent removal from isotropic micellar solution, since (i) reconstitutions were shown to be slightly affected by the rate of detergent removal; (ii) step-by step reconstitutions allowing direct incorporation into preformed liposomes destabilized by octyl glucoside or Triton X-100 were found much more efficient than reconstitutions from micellar solutions (see Section 5.1).

Discussion of the model and comparison with available data

Regarding the effects of the rate of detergent removal, the model is in line with that previously reported by Eytan [8]; see also [67,68]. However, two important points demonstrated by our experimental results are that (i) the rate of detergent removal has to be controlled in the case of proteins which tend to form unstable ternary lipid-protein-detergent micelles; (ii) slow detergent removal leads to the formation of protein-rich structures prior to liposome formation and not to a sequence of events in which liposomes might be formed first. In other words, detergent is removed preferentially from unstable ternary micelles than from binary lipid-detergent micelles.

Concerning the state of aggregation of the proteins at the detergent concentration where membranes begin to form from the solubilized lipids and its role in determining the sequence of events for proteoliposome formation, the model we propose is in line with that reported by Helenius et al. [69]. These authors analyzed the reconstitution of the Semliki-Forest-virus spike glycoprotein which exists as two complexes corresponding to the monomeric and oligomeric forms of the protein. When these complexes were reconstituted through octyl glucoside dialysis or dilution, three types of products were obtained: the monomeric proteins were found to be incorporated in vesicles at the detergent concentration at which the lipid bilayers form; the protein molecules present as oligomers associated at lower detergent concentrations by incorporation into preformed detergent-doped liposomes; variable amounts of lipoprotein complexes were obtained corresponding to non-incorporated proteins. They also evidenced that membrane penicillinase was oligomeric at the octyl glucoside concentration where the reconstitution occurred and proteoliposomes resulted from direct incorporation of the oligomeric protein into preformed liposomes. Studies by Curman et al. [89] on the asymmetric incorporation of HLA antigens also supported this model of lipid-protein association in the presence of octyl glucoside. We also demonstrated that spontaneous insertion of crystalline arrays of bacteriorhodopsin [24] or of string-like structures of H^+-ATPase (unpublished results) occurred in octyl-glucoside-saturated liposomes.

How the insertion of the proteins into preformed liposomes destabilized by saturating amounts of detergent occurs is not clear. In all previous instances where spontaneous incorporation of integral membrane proteins have been analyzed, the data showed that phospholipid bilayers might have the property that is required for spontaneous fusion. Indeed, direct insertions of membrane proteins were achieved in the presence of cholesterol, fatty acids, lysophospholipids or detergents, and have also been reported to depend upon the state of the bilayer, the size of the liposomes and phospholipid composition [8,14,48–57,93,94]. Jain and Zakim [14,93,94] have proposed that the putative role of these impurities was the formation of organizational defects in the bilayers that might act as sites of fusion of vesicles with other vesicles, but also for fusion of vesicles with aggregates of proteins. However, such mechanisms do not seem to apply in our experimental conditions, since (i) the interaction of proteins with octyl-glucoside-saturated liposomes is a rapid and random process, being complete in very few minutes; (ii) the association of the protein with preformed liposomes occurs independently of fusion between large unilamellar liposomes as shown by electron microscopy. Thus, the mechanisms we could propose are more in line with those already suggested by Helenius et al. [69]. It could be that insertion of protein oligomers occurs via the monomeric form and would depend upon the stability of protein oligomers and/or on the efficiency of the detergent to dissociate the oligomers. In this framework, Christiansen and Carlsen [95] by analyzing the mode of cytochrome b_5 reconstitution through the use of different methods, concluded that when cytochrome b_5 aggregates were added to preformed liposomes, only cytochrome b_5 in a monomeric state could be inserted into liposomes in a mode not susceptible to attack by carboxypeptidase Y. They pointed out for an efficient dissociation of aggregated cytochrome b_5 into monomers by lysoderivatives as compared to other detergents. In view of our results, octyl glucoside, which has a very high cmc, would be a very efficient detergent in terms of oligomer dissociation and monomer insertion. On the other hand, Triton X-100 would be less efficient, since direct incorporation is observed to occur in the presence of an amount of detergent exceeding that needed for liposome saturation to allow the dissociation of protein oligomers and/or their further incorporation into liposomes. In the case of the Ca^{2+}-ATPase, due to the high propensity for self-association of this protein, the size and/or stability of protein oligomers might be so great that they could not be dissociated by Triton X-100 and that consequently very few or heterogeneous proteoliposomes were formed.

5. A new efficient reconstitution procedure

5.1. A step by step reconstitution procedure

Besides providing information about the way by which proteins may associate with phospholipids during deter-

gent-mediated reconstitutions, we believe that an important advance contributed by our studies is the finding that the strategy described is a method of choice for membrane protein reconstitution and is more suitable than the methods previously used.

The advantage of the method described in this review, involving protein incubation in detergent-treated liposome suspensions at each step of the solubilization process, is to allow a 'snapshot' on all situations that may occur in an usual detergent-mediated reconstitution. For comparison, the almost standard procedure for detergent-mediated reconstitution consists of co-solubilizing the membrane proteins and lipids with detergents to form a suspension of mixed micelles and then removing the detergent. Because phospholipid and protein solubilities are generally small, detergent removal that starts from lipid and protein in the same micelle can be expected to lead to the formation of proteoliposomes with a lipid-to-protein ratio similar to that in the initial ternary micelle. The consequence is that, even if a protein can be inserted into a preformed liposome, this step may be missed during detergent removal from micellar solutions, since proteoliposomes can be readily and preferentially formed from the ternary phospholipid-detergent-protein micelles initially present. This has been clearly evidenced in the case of reconstitution of bacteriorhodopsin using octyl glucoside or Triton X-100: much higher initial rates and total extent of proton pumping were observed for the samples reconstituted from starting detergent to phospholipid ratios below those necessary for complete solubilization of the initial material (see Table 4). In the same framework, reconstitutions of F_0F_1-ATPases and co-reconstitutions of bacteriorhodopsin F_0F_1-ATPases are much more efficient when using the step-by-step reconstitution procedure with octyl glucoside or Triton X-100 than when starting from totally solubilized samples.

However, the greater efficiency of the step-by-step procedure appears less clear in the case of the reconstitution of proteins with a tendency for self-aggregation. For example, in octyl-glucoside-mediated reconstitutions of Ca^{2+}-ATPase, very similar activities were measured in proteoliposomes reconstituted at the onset of solubilization or from totally solubilized samples [28]. Indeed, according to the model proposed for this protein (Fig. 8, scheme A), preformed liposomes and lipid-protein complexes may coexist during the course of detergent removal from a mixture of binary and ternary micelles both structures. Thus, if the detergent still present has the ability of incorporating the 'aggregated' protein into the preformed liposomes, no

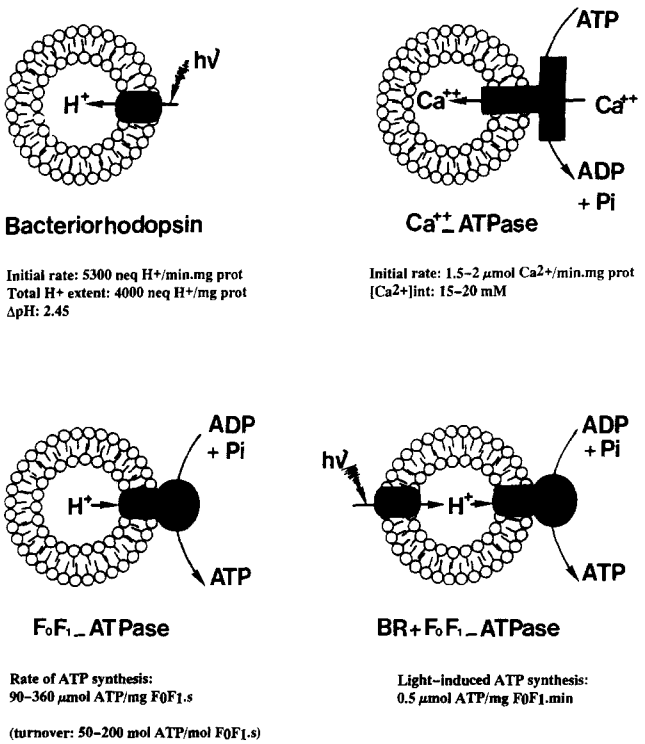

Fig. 9. Efficiency of optimal detergent-mediated reconstitutions of different membrane proteins. Light-induced protons movements in bacteriorhodopsin-containing proteoliposomes were assayed by the pH meter, [^{14}C]methylamine distribution and changes in fluorescence of the pH-sensitive probe pyranine (Refs. [23,40–44]). Ca^{2+} uptake by Ca^{2+}-ATPase proteoliposomes were measured in the absence of oxalate and in the presence of a protonophore by using murexide to monitor external Ca^{2+} concentration (Refs. [25,27]). ATP synthesis by F_0F_1-ATPase proteoliposomes were measured through artificially imposed $\Delta pH/\Delta\psi$ gradients either after 15 s incubation in the basic medium (ATP yield) or from initial rates of ATP synthesis (ATP turnovers). See Ref. [26]. Light-induced ATP synthesis by proteoliposomes co-reconstituted with bacteriorhodopsin and F_0F_1-ATPases from different sources (chloroplasts, mitochondria or PS_3 thermophilic bacillus) were measured using the luciferin-luciferase assay.

large difference in the resulting proteoliposomes is expected, whether reconstitution started from totally solubilized samples or from direct incubation of the protein with preformed liposomes.

The other advantage of the method described in this review is to allow fast and easy determination of the experimental conditions for optimal detergent-mediated reconstitution of any protein. We already know that it is useful for reconstitution of bacteriorhodopsin, sarcoplasmic reticulum Ca^{2+}-ATPase and chloroplast F_0F_1-ATPase. Fairly high activities were observed for these three proteins with very different structural properties, thus proving the efficiency and general validity of the procedure in creating highly functional proteoliposomes. Furthermore, we have been recently able to obtain successful reconstitution of purified human erythrocyte plasma membrane Ca^{2+}-ATPase [30] and of *Synechocystis* cyanobacterial Photosystem I (unpublished results). Using similar strategy and favorable detergents, efficient reconstituted proteoliposomal systems were obtained with myometrial ocytocin receptor [86] and band-3 purified from human erythrocyte membrane (Boulter, J., personal communication). Finally, we recently demonstrated that this strategy, efficient for reconstitution of individual proteins, was very successful for the co-reconstitution of two energy-coupled proteins: co-reconstitution of proteoliposomes with bacteriorhodopsin and F_0F_1-type H^+ATPases from different sources (chloroplasts, mitochondria, PS_3 thermophilic bacteria) yields vesicles which are able to sustain high ATP synthesis activities upon light activation of bacteriorhodopsin (our unpublished data).

It is noteworthy that the activities of the reconstituted proteoliposomes are among the best reported up to now for each of these proteins, and for many of them relevant to physiological conditions (Fig. 9). For comparison, one can refer to previously reported reconstitution studies on bacteriorhodopsin [9,10], Ca^{2+}-ATPase [16,96–100], F_0F_1-ATPases [88,101–103] and bacteriorhodopsin-F_0F_1-ATPases[101,104–112].

5.2. Protein orientation

Another important aspect of our study was that the mechanism by which proteins associate with phospholipids to give proteoliposomes was shown to have a critical effect on the final orientation of the protein into the bilayer (Table 4; [23,25,26,28]). Better asymmetrical orientations and consequently higher activities were always observed for the samples reconstituted by incorporation of the protein into preformed liposomes. Optimal unidirectional orientations (85 to 95%) were obtained after direct protein incorporation into octyl-glucoside-saturated liposomes whatever the protein analyzed. Although less efficient, good asymmetrical orientations (70 to 80%) were also observed in Triton X-100-mediated reconstitutions in which, the starting detergent-lipid mixtures contained a large amount of preformed detergent-doped liposomes.

Note that these results support the idea that the insertion of a protein into preformed liposomes leads to the formation of proteoliposomes with a better asymmetrical protein insertion than when they are formed by detergent removal from ternary phospholipid-detergent-protein micelles [8,14]. One possible mechanism explaining the unidirectional orientation of a membrane protein when it is incorporated into preformed liposomes is that protein is always inserted through the hydrophobic domain of the membrane with its most hydrophobic moiety first. This seems obvious for asymmetrical membrane proteins such as Ca^{2+}-ATPase or F_0F_1-ATPase. In the case of bacteriorhodopsin, its carboxylic tail is the most hydrophilic, as it contains at least five COOH groups, while the NH_2-terminal region is the most hydrophobic. Thus, the latter will be the first to be inserted into the membrane, leading to almost inside-out

Table 4
Comparison of the H^+ pumping efficiencies of bacterioorhodopsin proteoliposomes reconstituted from Triton X-100, octyl glucoside or sodium cholate

Detergent	ΔpH	Total H^+ (neq H^+)	Initial rate (neq H^+/min)	Inside out (right side out) orientation %	Size (nm)
Triton X-100 (partial solubilization)	2.2	520	430	80–85 (20–15)	160
Triton X-100 (total solubilization)	1.65	345	220	65–70 (35–30)	200
Octyl glucoside (onset solubilization)	2.45	400	530	95 (5)	100
Octyl glucoside (total solubilization)	1.9	300	370	70–75 (30–25)	200
Sodium cholate (total solubilzation)	2.2	150	360	70–75 (30–35)	70

Liposomes were incubated in the presence of bacteriorhodopsin and different amounts of detergent corresponding to either total solubilization (stage III), to partial solubilization (stage II) or to the onset of solubilization (end of stage I). After detergent removal, H^+ pumping activities [24] were measured by the pH-meter technique (the values indicated in the table correspond to 100 mg of protein), by fluorescence quenching intensity of entrapped pyranine and by distribution of weak bases by flow dialysis. The percentage of inside-out orientation of bacteriorhodopsin was determined from the effects of one-sided inhibitors on the light-induced fluorescence changes of pyranine. Size of the proteoliposomes derived from distribution histogramms by freeze-fracture electron microscopy.

orientation of bateriorhodopsin in the resulting proteoliposomes. Interestingly, in the case of direct incorporation of cytochrome *c* oxidase, it was shown [51] that if the charge of the liposomes played a role in the initial binding of this enzyme to preformed liposomes (the enzyme did not bind efficiently to neutral liposomes) it played no role in the orientation of the enzyme, which was always found with its cytochrome *c* binding site facing outward in the liposomes. This clearly demonstrated that the determining factor in the unidirectional orientation of cytochrome *c* oxidase was the relative hydrophobicity of the various moities of this protein, the cytochrome *c* binding being the most hydrophilic.

If there is general agreement about the unidirectional orientation of membrane proteins following direct incorporation, the discussion is more open concerning the final orientation when forming proteoliposomes by detergent removal from micellar solutions. If random orientations (50%) have generally been reported, preferential orientation of membrane proteins have been reported in many instances after detergent dialysis [8–10,16,17]. Other parameters, such as the respective charges of the proteins and lipids, or steric factors, should be taken into account when considering the final orientation of the protein. In this context, the functional orientation of reconstituted cytochrome *c* oxidase has been demonstrated to considerably depend upon the electrostatic interactions between the protein and the phospholipids using the cholate dialysis method [113–115]. Transformation of the protein charge by specific reagents or use of negatively charged phospholipids [113] shown that the positive charges of the matrix side determined the dominant right-side out orientation of this strongly dipolar enzyme [116].

5.3. Use of SM_2 Bio-Beads for detergent removal

Besides the advantage of allowing rapid determination of the optimal reconstitution conditions, the procedure described in this review is also advantageous for its method of detergent removal. Efficient removal of detergent from the reconstituted proteoliposomes is an absolute necessity, because residual detergent may either inhibit enzyme activity and/or drastically increase the passive permeability of liposomes [22,27]. The method we used to remove detergent was based on the procedure originally described by Holloway [57], namely detergent adsorption onto hydrophobic SM_2 Bio-Beads. Using these polystyrene beads, we showed that Triton X-100, octyl glucoside, cholate and $C_{12}E_8$ could be removed efficiently [23,24,27]; recent studies with Chaps, Chapso and dodecylmaltoside confirm the general use of Bio-Beads to remove all kinds of detergents (our unpublished results). Systematic studies demonstrated the importance of the initial detergent concentration, the amount of beads, the nature and commercial source of detergent, the temperature and the presence of phospholipids, in determining the rate of detergent adsorption onto Bio-Beads. One of the main findings of our studies was that Bio-Beads allowed almost complete removal of detergents whatever the initial experimental conditions. This is particularly important for detergents with a low critical micelle concentration such as Triton X-100 and $C_{12}E_8$ (reconstitutions mediated by these detergents led to vesicles containing in any case less than 7 detergent molecules/100 lipids). Furthermore, we demonstrated that all the inherent drawbacks of the methods reported in the literature (high residual detergent, large loss of lipids and formation of multilamellar structures) could be minimized or overcome [27].

Another important positive benefit of our reconstitution method is to provide a suitable reproducible way of obtaining, large, unilamellar and fairly homogeneous liposomes, starting from a micellar solution. The principles of the procedure are as follows: (i) enough detergent must be added for complete lipid solubilization; (ii) the initial Bio-Bead concentration must be adjusted so as to promote micellar-to-lamellar transition in not less than about 3 h; (iii) after this transition, the Bio-Bead concentration can be increased to accelerate detergent removal and; (iv) the final Bio-Bead concentration must never exceed about 50-times the lipid concentration (w/v). Among the main parameters affecting the results of the reconstitution (temperature, Bio-Bead-to-detergent ratio, etc...), we believe that the most important is the low bead-to-detergent ratio used in the first step of reconstitution: this is essential, both to avoid phospholipid losses and to allow slow detergent removal during the micellar-to-lamellar transition, thus giving homogeneous and relatively large liposomes. In this connection, freeze-fracture electron microscopy studies demonstrated that the rate of detergent removal critically affected the final size distribution of the reconstituted liposomes. Small unilamellar liposomes are formed by fast detergent removal, whereas larger liposomes are formed by slow removal [24,27].

Finally, we would like to point out a more technical feature which emerges from our study and which specifically concerns membrane protein reconstitution into liposomes: it is that, as pointed out in a recent workshop on membrane protein reconstitution [117], the rate of detergent removal is a key factor for final protein orientation, lipid-protein ratio homogeneity and/or liposomal morphology. Therefore, useful detergent-mediated reconstitution procedures must be capable of varying and controlling the rate of detergent removal: on the basis of our results, this is clearly the case for the batch procedure using SM_2 Bio-Beads as the detergent removing agent, whatever the detergent. As a representative example, the functional reconstitution of Ca^{2+}-ATPase in the presence of Triton X-100 or $C_{12}E_8$ was shown to be highly dependent on the rate of detergent removal: due to the propensity for self-aggregation of this protein upon slow detergent removal, production of proteoliposomes with high Ca^{2+} transport activities required rapid detergent removal [28].

5.4. Low passive permeability

One indication of almost complete detergent removal is the relatively low permeability of the reconstituted proteoliposomes, whatever the detergent used. In particular, the ion passive permeability of proteoliposomes containing energy-transducing membrane proteins such as bacteriorhodopsin, Ca^{2+}-ATPase or H^+-ATPase is an important parameter in active transport processes, because it partially determines both the range of attainable $\Delta\mu H^+$ or $\Delta\mu Ca^{2+}$ values and the rates of proton gradient-driven ADP phosphorylation by H^+-ATPases. This is why ion passive permeability was investigated in detail in all the reconstituted systems studied in our laboratory. The proton and counterion fluxes generated by external acid pulses were monitored, using the fluorescence of the pH-sensitive probe pyranine trapped inside reconstituted liposomes [24,27, 43,44]. Permeabilities to anions and cations were systematically measured as a function of the ionic composition of the medium, temperature, the presence of ionophores (valinomycin and protonophores) and lipid-to-protein ratios. The most striking feature of our studies is that the proteoliposomes obtained by the strategy described in this paper are relatively tight as they are impermeant to SO_4^{2-}, PO_4^{2-} and Ca^{2+}, and slightly permeant to H^+, K^+ and Na^+. The observed H^+ permeabilities of the liposomes prepared by our method (containing a mixture of egg PC and egg PA, 9:1 mol/mol) were in the range of 10^{-5} cm s^{-1} larger than those of the liposomes prepared by reverse-phase evaporation [43,44], but were still much smaller than those of other liposomal preparations [118].

Such low permeabilities are clearly an improvement in the reconstitution procedures, especially when studying the functional characteristics of energy-transducing membrane proteins. Among the relevant advantages of the proteoliposomes reconstituted during the course of our studies which can be extended to the studies of other energy-transducing membrane proteins one can cite:

(1) Accumulation of massive amounts of H^+ by bacteriorhodopsin-containing liposomes, leading in the presence of valinomycin to light-induced steady-state pH gradients of 2–2.5 pH units at 25°C, which are among the highest values reported to date for such proteoliposomes (for comparison see [9,10]). Due to the high value of ΔpH, we have been able to clearly evidence the ultimate consequence of the retro-inhibitory ('back pressure') effect of ΔpH upon the functioning of bacteriorhodopsin [44]. Another advantage of the low ionic permeability relies also on the possibility to analyze the retroinhibitory effect of an electrical potential, $\Delta\psi$, on bacteriorhodopsin functioning [119]: indeed, in the absence of valinomycin, low proton pumping activities were observed which could be stimulated by a factor of 10 by the antibiotic which overcame, by compensatory K^+ movements, the inhibitory effect of the $\Delta\psi$ [23,44].

(2) High steady-state Ca^{2+} accumulation [25,27,29,30], corresponding to lumenal concentration of approximately 20 mM, by Ca^{2+}-ATPase proteoliposomes, without the need of precipitating agents such as oxalate or phosphate.

(3) Demonstration that the Ca^{2+}-ATPases from sarcoplasmic reticulum and red blood cells behave as electrogenic Ca^{2+}/H^+ exchangers [25,29,30]. It was clear from direct pH measurements and stimulation of Ca^{2+} transport by protonophores that Ca^{2+} transport was accompanied by countertransport of H^+ and that consequent alkalinization of the lumen of the proteoliposomes hindered Ca^{2+} transport. With regard to the mechanisms of Ca^{2+}/H^+ countertransport, a direct 1:1 Ca^{2+}/H^+ exchange was demonstrated. The net positive charge transfer and the electrical potential detected by the oxonol probe were consistent with the 1:1 stoichiometric Ca^{2+}/H^+ ratio and the estimated charge transfer. The compensation of the $\Delta\psi$ by different anions and cations have been further analyzed in detail [120]. More recently, the effect of lumenal and extravesicular pH variations on the Ca^{2+}/H^+ stoichiometries has been reported, reflecting the protonation and dissociation behavior of acidic residues involved in cation binding and translocation by the sarcoplasmic reticulum Ca^{2+}-ATPase reconstituted into liposomes[121].

(4) Rates of ATP synthesis driven by artificially imposed $\Delta pH/\Delta\psi$ transitions were shown constant up to 10–20 s[26] as compared to 200 ms in previously reported reconstitution studies of chloroplast F_0F_1-ATPase [102,103]. Such a property permitted analysis of some aspects of ATP synthesis regulation without the need of rapid mixing techniques [112].

(5) Light-induced ATP synthesis by proteoliposomes co-reconstituted with bacteriorhodopsin and F_0F_1-ATPase from different sources (chloroplasts, mitochondria, thermophilic bacillus PS3) were in the range of the highest values reported to date (for comparison see [104–112]). In particular, the correlations between rates of ATP synthesis by thermophilic ATPases and light-induced H^+ flux were investigated at 40°C under different experimental conditions. At saturating light-intensities, maximal rate of ATP synthesis up to 500 nmol ATP/min per mg protein were obtained (i.e., 20-times higher than those previously reported for the reconstitution of the same ATPase; [105,106]) and found to be constant for several hours.

5.5. Lipid-to-protein ratio

Another important parameter to control and to vary in reconstitution experiments is the lipid-to-protein ratio. In this context, this new reconstitution strategy was shown to be efficient for total and homogeneous protein incorporation within a large range of lipid-to-protein ratios (from about 160 to 5 (w/w) for most proteins analyzed). Among the advantages to optimize the density of protein molecules in the reconstituted membranes one can cite:

(1) Extensive dilution of pumping units in the lipid bilayers. This allows in Ca^{2+}-ATPase proteoliposomes

(lipid-to-protein weight ratio of 80, i.e., about 15 protein molecules per vesicle), transport of large amounts of Ca^{2+} through repeated enzyme cycles over a relatively long time of experimental observation. Under optimal conditions maximal levels ranging between 3 and 10 μmol Ca^{2+}/mg Ca^{2+}-ATPase were reached in 15–20 min, corresponding to a lumenal concentration of 20 mM calcium or higher. As a term of comparison, Ca^{2+} uptake by native sarcoplasmic reticulum vesicles (lipid-to-protein ratio: 1:1) reaches asymptotic levels of about 100 μmol Ca^{2+}/mg protein (i.e., about 15–20 mM internal Ca^{2+}) in about 90 s, after which further transport is inhibited by the lumenal Ca^{2+} concentration rise. Thus, the experimental advantage of the proteoliposomes is related to the prolonged time required to reach the inhibitory level of lumenal Ca^{2+}, due to the high volume available per unit protein. Another important consequence of the high volume available per Ca^{2+}-ATPase is that Ca^{2+} accumulation can be studied without the need of calcium precipitating agents such as oxalate or phosphate: the ultimate consequence is that the initial rates of Ca^{2+} uptake were found higher in the proteoliposomes than those measured in native vesicles in the presence of oxalate [25]. This is consistent with the good asymmetric orientation of the Ca^{2+}-ATPases in the proteoliposomes and with a delayed Ca^{2+} uptake in sarcoplasmic reticulum vesicles due to Ca^{2+}-oxalate precipitation.

Interestingly also, due to the favorable ratio of lumenal volume per ATPase unit, the Ca^{2+}/ATP coupling ratio could be monitored accurately during the very slow rise in lumenal Ca^{2+} concentration. It has been clearly evidenced [122] that this ratio decreased gradually as lumenal Ca^{2+} increased, leading the authors to propose branched pathways in the reaction scheme of the Ca^{2+}-ATPase.

(2) Optimization of the ATP yield in F_0F_1-ATPase proteoliposomes. The ATP yield measured 30 s after a $\Delta pH/\Delta\psi$ transition was shown to be optimum at an average distribution of one CF_0F_1/vesicle. If the enzyme concentration was doubled, the ATP yield dropped by a factor of 2, since the same amount of internally buffered protons was now distributed between two enzymes.

(3) Possibility to analyze in detail the inhibitory control of energy-transducing membranes by the ionic gradients they generate. Proton pumping activities by bacteriorhodopsin proteoliposomes were found to increase linearly for lipid-to-protein ratios ranging from 10 to 160 (w/w). On the other hand, the steady-state pH gradients appeared independent of the number of pumping units in the membranes of the proteoliposomes. Such experiments clearly showed that bacteriorhodopsin proteoliposomes did not behave as simple pump and leak systems, giving pieces of evidence for a retro-inhibitory effect of ΔpH on bacteriorhodopsin functioning [23,44]. Similar studies on Ca^{2+}-ATPase proteoliposomes reconstituted at different lipid-to-protein ratios demonstrated the inhibition of the Ca^{2+}/H^+-ATPases by either the internal Ca^{2+} concentration, or the internal alkalinization and confirmed that the level of accumulated Ca^{2+} was directly related to the final alkalinization of the intraliposomal space and reciprocally [25,28,30].

(4) Optimization of the rates of light-induced ATP synthesis in bacteriorhodopsin-F_0F_1 proteoliposomes. Optimal co-reconstitutions of these two functionally coupled proteins were shown to require a high content of H^+-generators but a low content of H^+-consummers. Furthermore, the possibilities to vary the lipid to bacteriorhodopsin ratio, i.e., the vicinity between $\Delta\mu H^+$ consumers and $\Delta\mu H^+$ generators, allowed us to investigate the relative importance of localized versus delocalized coupling phenomena (unpublished data).

(5) Oligomerization/two-dimensional crystallization. Reconstitutions at low lipid-to-protein ratios (i.e., below 10 (w/w)), although not very useful for functional purposes, due to a large increase in membrane permeability of the resulting proteoliposomes, were found to be a powerful tool for structural purposes. In particular, we have been able to demonstrate through freeze-fracture electron microscopy studies the occurrence of a lipid-to-protein ratio-dependent bacteriorhodopsin monomer-oligomer equilibrium [40]. Our results showed unambiguously that bacteriorhodopsin was in a monomeric form at high lipid-to-protein ratios (40 w/w) and started to self-associate at lower lipid-to-protein ratios to form well-defined oligomers (trimers) which were the sole species present at lipid-to-protein ratio of 1 w/w. It is likely that the formation of such oligomers may be important as the first steps in the formation of two-dimensional crystals of bacteriorhodopsin. More recently, we have been able by reconstituting Ca^{2+} ATPases at lipid-to-protein ratios of about 1 (w/w) (collaborations with D. Stokes) to reproduce in proteoliposomes the vanadate-induced two-dimensional crystallization of Ca^{2+}-ATPase previously observed in native sarcoplasmic reticulum vesicles [123]. This leads to interesting perspectives in the study of the crystallization process of P-type ATPases through the use of well-defined proteoliposomes of different compositions.

5.6. Other comments

Other factors of importance for membrane protein reconstitution which can be foreseen from our experimental studies are related to:

(1) Pre-solubilization of the protein before reconstitution to ensure a predominantly monomeric state. Although it has been possible to reconstitute bacteriorhodopsin in the form of purple membrane sheets through direct incorporation into octyl-glucoside-saturated liposomes, this incorporation was limited to a final lipid-to-protein ratio of 80 (w/w) as compared to lipid-to-protein ratios up to 1 (w/w) with monomeric bacteriorhodopsin. With other detergents, efficient reconstitution showed an absolute requirement for a monomeric state of bacteriorhodopsin

([23]; see also [111]). In the same framework, it was found essential to ensure complete presolubilization of F_0F_1-ATPases and Ca^{2+}-ATPases for efficient reconstitution.

(2) Sequential addition of proteins, detergents and phospholipids. The order of addition has been shown to affect drastically the biological activity of the resulting proteoliposomes and has to be adapted to the specific interaction of proteins with detergents. For example, octyl glucoside caused immediate and irreversible inactivation of Ca^{2+}-ATPase activity when added to sarcoplasmic reticulum vesicles or to $C_{12}E_8$-presolubilized purified protein. However, inactivation can be delayed to hours in the presence of phospholipids. Thus for octyl-glucoside-mediated reconstitution of Ca^{2+}-ATPase the strategy was first to add the desired amount of octyl glucoside to preformed liposomes and then add the $C_{12}E_8$-presolubilized active enzyme. In these conditions the inactivation of the protein by octyl glucoside is overcome and the most efficient proteoliposomes can be produced [25,27]. In contrast, for reconstitution of F_0F_1-ATPases, due to the presence of aggregates or stings in solubilized and purified preparations of these proteins, it was found more efficient to add first the desired amount of detergent to the protein and then add this highly concentrated detergent solution to preformed liposomes.

Another advantage of our strategy is related to the possibility to reconstitute a protein purified and presolubilized in a specific detergent with liposomes treated by another detergent. For reconstitution at high lipid-to-protein ratios the small amount of detergent added together with the protein did not affect results of reconstitution mediated by an excess of another detergent.

(3) The nature of the phospholipids used. It is well established that the purity of the phospholipids used for reconstitution is a key factor in determining the efficiency of a reconstitution. These phospholipids must (i) form well-sealed proteoliposomes and (ii) provide favorable lipid-protein interactions for biological activity. Mixtures of egg phosphatidylcholine and egg phosphatidic acid appeared along our studies of different membrane proteins to fulfil these conditions. In particular asolectin (a mixture of phospholipids extracts from soy bean) gave irreproducible and unsatisfactory results as compared to pure phosphatidyl choline from egg yolk: asolectin proteoliposomes were found to be much more permeant and consequently displayed much lower biological activities. On the other hand, the presence of negatively charged phospholipids (10–20% egg phosphatidic acid) led to bacteriorhodopsin , Ca^{2+}-ATPase and bacteriorhodopsin-F_0F_1 ATPases proteoliposomes with significantly higher activities without perturbing protein incorporation, protein orientation and vesicle size. In this context, it must be kept in mind that the choice of a particular lipid to use in reconstitution must be made carefully and that in any case the role of a lipid in preservation or activation of the biological activity must be distinguished from indirect effect on the reconstitution process (structure, size and permeability of proteoliposomes or protein incorporation and orientation).

6. Conclusions and perspectives

The reconstitution of membrane proteins into liposomes has proved to be, and should remain a potentially powerful tool that can be used to identify the mechanism of action of membrane proteins. As a prerequisite, sound characterization of the reconstitution method is required for proteoliposome reconstitution to be optimal. As shown by this short review, the prospects of achieving this are obviously good, provided the experimental analysis is systematically performed. Four conclusions worth mentioning emerge from our results:

(1) Three mechanisms of lipid-protein associations were evidenced and primarily related to the nature of the detergent used for reconstitution: proteins can be inserted into detergent-saturated liposomes (octyl-glucoside-mediated reconstitution), they may simply participate in the proteoliposome formation during the micellar-to-lamellar transition (cholate-mediated reconstitution), or they can be transferred from mixed micelles to detergent-saturated liposomes (Triton X-100-mediated reconstitution).

(2) Although the products of reconstitution clearly depend mainly on the particular detergent used, the tendency towards self-association and/or aggregation of the protein can be a key factor in determining the composition of the final proteoliposomes.

(3) The final orientation of the protein in the membrane is critically dependent upon the mode of protein-lipid association. Proteins were found to be more asymmetrically oriented in the reconstituted liposomes when they were integrated into preformed liposomes than when they were integrated during proteoliposome formation by detergent depletion of ternary mixed micelles.

(4) The strategy we used to study the mechanism of protein incorporation proved to be a powerful reconstitution procedure which was more effective than the usual methods using detergents. It allowed a 'snapshot' on each step in the lamellar-to-micellar transition, thus permitting rapid and easy determination of the optimal reconstitution conditions for any membrane protein. An additional advantage of this reconstitution procedure relies on the batch procedure using SM_2 Bio-Beads as the detergent-removing agent, because this provides an easy reproducible way of obtaining unilamellar, relatively large and impermeable vesicles. The method therefore produced proteoliposomes which satisfy most of the criteria for efficient reconstitution, with the highest sustained transport activities reported to date and for many of them, comparable to those measured in native membranes.

However, it should be stressed from our results that the underlying processes which lead to the formation of specific reconstituted forms of liposomes are not well under-

stood. During the last decade, a good deal of information has been published concerning the processes of liposome solubilization, as well as the mechanism of vesicle formation upon detergent depletion. It is to be hoped that the gradual accumulation of such information, together with future detailed studies of the kind described in this review, will result in the formulation of a general set of principles which would serve as a guide in the elaboration of reconstitution experiments.

The future of membrane protein reconstitution appears bright in the light of the steadily expandable number of membrane proteins which can be expressed using molecular-biological methods. Powerful methods of over-expression of genes in bacteria and eukaryotic cells are now available, resulting in the production of interesting membrane proteins in quantities sufficient for reconstitution trials. Furthermore, the availability of mutated proteins renders efficient and reliable methods for reconstituting them as a need for further progresses in understanding their structure–function relationships.

Further work of interest should include detailed studies of the reconstitution process through the use of new detergents and other membrane proteins. New information from structure, thermodynamics and dynamic properties of lipid-protein-detergent micelles would be of prime importance. They might considerably refine the reconstitution methods by giving a clear picture of the formation of proteoliposomes from micellar structures and information about the molecular mechanisms by which proteins reassemble with phospholipid bilayers.

Finally, of primary interest also would be investigations directed towards two-dimensional crystallization of membrane proteins. An increasing number of membrane proteins have been assembled into crystalline sheets, vesicles and tubes (for recent reviews see [124–128]). High-resolution structural data of two-dimensional crystals have been collected for several membrane proteins, and electron crystallography offers now a viable alternative to X-ray crystallography for structure determination at high resolution. However, in spite of several advances in two-dimensional crystallization with near-atomic resolution, it is still the case that a lot of two-dimensional crystals are limited to medium or low resolution. This drawback is obviously related to the quality (order, size) of the two-dimensional crystals which in turn is related to the very empirical approach of the two-dimensional crystallization experiments. Since the most universal method of two-dimensional crystallization is based on removal of detergent from micellar lipid-protein-detergent solution, the general concepts developed in this review on membrane protein reconstitution at relatively high lipid-to-protein ratios should be transferred to reconstitutions at lower lipid-to-protein ratios. In the framework of the processes described in this review, the two-dimensional crystallization of a membrane protein by detergent removal from a micellar solution could be described as proposed by Kühlbrandt [124] as a one-, two- or three-stage process. In the three-stage model, binary-lipid-detergent and ternary lipid-detergent-protein micelles dissociate separately forming first lipid bilayers and lipid-protein complexes. During the second stage, the protein molecules insert into the preformed bilayers and during a third stage proteins arrange on a two-dimensional lattice (corresponding to scheme A, process 2 in the model proposed in Fig. 8). In the two-stage model, formation of the lipid bilayer and insertion of the protein occur simultaneously as the different micelles coalesce, followed by the stage of lattice formation (corresponding to scheme A, process 1 in the model proposed in Fig. 8). In the single-stage process, crystal contacts are established when micelles are detergent-depleted. Thus, to control and to optimize two-dimensional crystal formations, it appears of particular importance to analyze in molecular details the mechanisms of their formation by analysis of lipid-protein, lipid-detergent, protein-protein interactions but also by analysis of the different structures formed during detergent removal as well as their sequence orders.

In conclusion, there is much hope that with an increasing interest in studying lipid-protein-detergent interactions through the formation of well-characterized proteoliposomes or well-ordered two-dimensional crystals, our knowledge on the structure–function relationships of diverse membrane proteins will rapidly increase.

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