[4] Reconstitution of Membrane Proteins into Liposomes

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Introduction

Screening of the genomes of various organisms demonstrated that about 25% of the sequenced genes encoded strongly hydrophobic proteins, which are integrated into cell membranes.[1] This observation emphasizes the importance of membrane proteins in many biological processes essential for life. However, the complexity of most biological membranes makes it difficult to study these membrane proteins *in situ*. Therefore, purification from the native membrane and further reincorporation of a purified membrane protein into an artificial membrane continue to be crucial steps in studying the function and structure of these molecules. The necessity for reconstitution arises because many membrane proteins express their full activity only when correctly oriented and inserted in a lipid bilayer. In particular, reconstitution has played a central role in identifying and characterizing the mechanisms of action of membrane proteins with a vectorial transport function.[2–4] More generally, through biochemical and biophysical approaches, it has led to important information about lipid–protein and protein–protein interactions as well as topological and topographical features of different classes of membrane proteins. The reconstitution of membrane proteins to form two-dimensional crystals confined in a membrane has led to important high-resolution structural information by electron crystallography.[5,6]

In many instances the ability to investigate membrane proteins through the use of reconstituted systems has long been limited by the fact that methods for producing high-quality proteoliposomes have not advanced in step with biochemical, biophysical, and molecular biology techniques. Thus, one of the limiting factors in obtaining molecular information is related to the lack of reproducible methods of reconstitution. Therefore, enormous efforts have been required to understand the mechanisms of reconstitution and for new approaches to be evaluated, refined, and applied

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[2] G. D. Eytan, *Biochim. Biophys. Acta* **694,** 185 (1981).
[3] E. Racker, *Methods Enzymol.* **55,** 699 (1982).
[4] J. L. Rigaud, B. Pitard, and D. Lévy, *Biochim. Biophys. Acta* **1231,** 223 (1995).
[5] W. Kühlbrandt, *O. Rev. Biophys.* **25,** 1 (1992).
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to available proteins in order to make reconstitution even more important as a tool for further structure–function relationship studies on membrane proteins.

This chapter deals with the various strategies commonly used to reconstitute proteoliposomes and focuses on approaches that have led to the production of highly functional proteoliposomes. General guidelines and rules are proposed in this area of study, which has long been viewed as more art than science.

Strategies for Reconstitution of Membrane Proteins into Liposomes

From the abundant literature concerning the insertion of membrane proteins into liposomes, four basic strategies can be outlined: mechanical means, freeze-thawing, organic solvents, and detergents. Although these reconstitution strategies have proved useful to prepare pure phospholipidic vesicles,[7] the additional insertion of a membrane protein during the reconstitution process has imposed many constraints that have hampered seriously their efficiency and applicability for proteoliposome reconstitution. Indeed, besides the need for conditions that preserve the activity of the protein, many criteria must be considered to fully optimize the reconstitution of a membrane protein: the homogeneity of protein insertion and its final orientation, the morphology and size of the reconstituted proteoliposomes, as well as their residual permeability.

For example, proteoliposome reconstitution by mechanical means, such as sonication, has been drastically limited because of local probe heating, which is difficult to control and leads to degradation and denaturation of many membrane proteins. In addition, the small size (10–20 nm) of the resulting proteoliposomes limits the internal volume in which transport membrane proteins can accumulate ions or solutes. In the same framework, organic solvents have been widely used to prepare liposomes with a high capture efficiency, using procedures such as solvent injection[8] and reverse-phase evaporation.[9] However, the usefulness of such strategies for reincorporating membrane proteins has been limited because organic solvents denatured most amphiphilic membrane proteins. Thus, the strategy has been limited to the reconstitution of only a few hydrophobic membrane proteins.[10,11] Even more drastic, many methods for preparing pure

[7] F. Szoka and D. Papahadjopoulos, *Annu. Rev. Biophys. Bioenerg.* **9,** 467 (1980)
[8] D. Deamer and A. D. Bangham, *Biochim. Biophys. Acta* **443,** 629 (1976).
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[10] A. Darszon, A. C. Vanderberg, M. H. Ellisman, and M. Montal, *J. Cell Biol.* **81,** 446 (1979).
[11] J. L. Rigaud, A. Bluzat, and S. Büschlen, *Biochem. Biophys. Res. Commun.* **111,** 373 (1983).

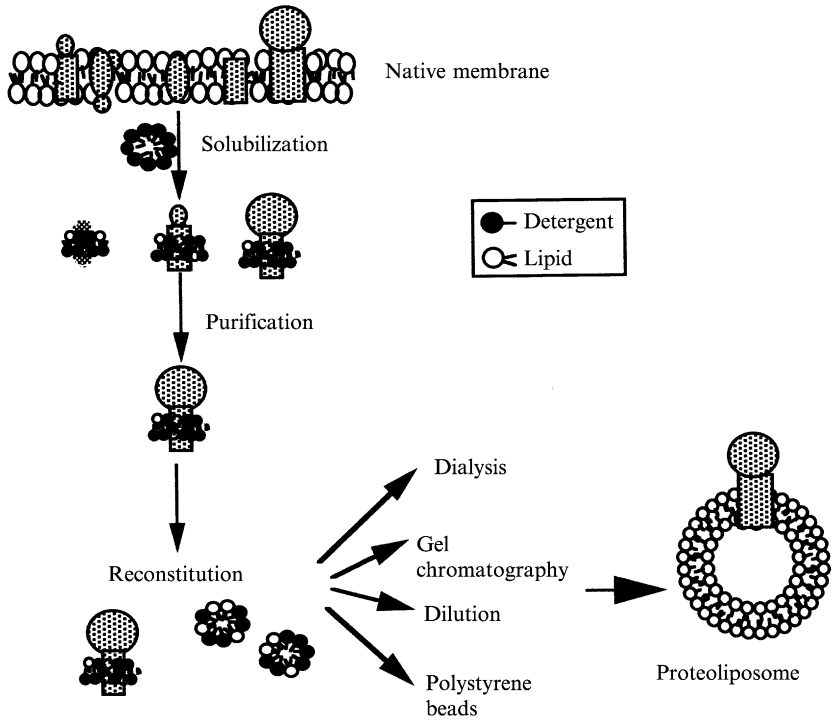

FIG. 1. Detergent-mediated reconstitution. Most membrane proteins are extracted from native membranes by solubilizing detergent concentrations. After purification, the solubilized protein is supplemented with an excess of lipids and detergent, leading to a solution of mixed lipid–protein–detergent and lipid–detergent micelles. For proteoliposome reconstitution, detergent is removed from these micellar solutions, using various strategies.

liposomes have been difficult to apply successfully to proteoliposome reconstitution because most membrane proteins are purified through the use of detergents, which interfere with the process of vesicle formation. Thus, the vast majority of membrane protein reconstitution procedures involve the use of detergents[2,4] (Fig. 1). Indeed, because of their amphiphilic character, most membrane proteins require detergents, not only as a means of disintegrating the structure of native membranes in the initial step of their solubilization, but also as a means of keeping the protein in a nondenaturing environment during further purification.[12]

The standard procedure in such reconstitutions involves comicellization of the purified membrane protein in an excess of phospholipids and

[12] M. LeMaire, P. Champeil, and J. Möller, *Biochim. Biophys. Acta* **1508,** 86 (2000).

appropriate detergent, to form a solution of mixed lipid–protein–detergent and lipid–detergent micelles. Next, the detergent is removed from these micellar solutions, resulting in the progressive formation of closed lipid bilayers in which the proteins eventually incorporate. All detergent-mediated reconstitutions described in the literature rely on the same standard procedure, differing only in the techniques used to remove the detergent.

Dialysis

Dialysis has been the most widely used method for detergent removal. Dialysis of mixed lipid–protein–detergent micelles against a detergent-free aqueous medium is based on the selective retention by a porous membrane of vesicles and micelles compared with detergent monomers. In dialysis experiments, only the detergent monomers diffuse through the dialysis bag and the rate of removal depends on the monomer detergent concentration across the bag. Detergents with high critical micelle concentrations (CMCs) such as octylglucoside, ionic detergents (sodium cholate, sodium deoxycholate), or 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) are easily removed by dialysis in 1–2 days; the technique is not appropriate for detergents with low CMCs such as polyoxyethylene glycols, which require 1–2 weeks for complete removal.[13]

In the simplest form of the method, the lipid–protein–detergent micellar solution is placed in a cellulose membrane bag with a cutoff of 14 kDa and dialyzed against a large volume of detergent-free aqueous buffer. Because of the necessity to scale down the amount of material for membrane proteins difficult to produce in large quantities, microdialysis devices have been used in the form of small compartments, allowing dialysis of 50- to 100-μl samples.[5,6]

The main advantages of dialysis are its simplicity and the low cost of the materials used. Also, the dialysis procedure generally leads to homogeneously sized vesicles. However, in its simplest setup, it suffers from numerous drawbacks, including (1) poor reproducibility, (2) an uncontrolled rate of dialysis, (3) possible retention of molecules on the dialysis membrane, and (4) duration of the experiments, which can be deleterious for many membrane proteins. Using a flowthrough dialysis cell can be advantageous as the rate of removal can be controlled carefully and the dialysis time decreased significantly.[14] Addition of polystyrene beads outside the

[13] T. M. Allen, A. Y. Romans, H. Kercret, and J. P. Segrest, *Biochim. Biophys. Acta* **601,** 328 (1980).

[14] M. H. W. Miloman, R. A. Schwender, and H. Weber, *Biochim. Biophys. Acta* **512,** 47 (1978).

bags, to maintain the external concentration of dialyzed detergent at zero, reduces the number of changes of buffer required during dialysis and can decrease the time of dialysis.[15] In conclusion, although the dialysis method has been applied successfully to many membrane proteins, it is unfortunately not well suited for detergents with low CMCs that require a long time for dialysis, which in turn is not always well tolerated by membrane proteins.

Gel Filtration

To avoid prolonged contact between proteins and detergent during reconstitutions, gel filtration has been used as a method for detergent removal. This strategy takes advantage of the different accessibility to the pores of a gel by mixed micelles as compared with liposomes. As the lipid–protein–detergent micellar mixture is eluted through the resin, the dilution resulting from both molecular diffusion and faster access of the smaller aggregates (monomers, mixed micelles) to the gel pore results in the formation of vesicles and their extrusion from the gel pores. The technique is simple, and gels of different pore size selectivity have been used in reconstitution experiments.[13]

The main advantage of this technique is its rapidity (5–10 min), avoiding long times of contact between detergent and protein. This, however, turns into a serious disadvantage in terms of incomplete and/or inhomogeneous protein incorporation and heterogeneous size distribution of the reconstituted preparations.[16] Thus, although it represents an interesting technique for preparation of pure liposomes, gel chromatography is no longer used in detergent-mediated reconstitution of membrane proteins.

Dilution

The dilution strategy is based on dilution by a detergent-free buffer of a lipid–protein–detergent micellar solution. The dilution must lower the initial detergent concentration to below its CMC, allowing spontaneous proteoliposome formation.

Cholate dilution was first employed for reconstitution. Lipids were mixed with membrane proteins at a final concentration of about 0.5% cholate, followed by a 25-fold dilution in the appropriate detergent-free buffer.[17] Other membrane proteins have been reconstituted by dilution

[15] J. Philippot, S. Mutaftschiev, and J. P. Liautard, *Biochim. Biophys. Acta* **734,** 137 (1983).
[16] M. Y. Abeywardena, T. M. Allen, and J. S. Charnock, *Biochim. Biophys. Acta* **729,** 62 (1983).
[17] E. Racker, T. F. Chien, and A. Kandrach, *FEBS Lett.* **57,** 1 (1975).

of octylglucoside micellar solutions.[18] The simplicity of the technique is evident, and the strategy has two main advantages that are related to (1) the short times required to decrease detergent concentrations and (2) the possibility of mastering the rate of dilution by progressive addition of the dilution buffer by syringe pumps powered either step by step or by synchronous motors. However, the technique suffers from numerous drawbacks that have made it unsuitable in many cases. Among them is the necessity to use detergents with high CMCs, because this limits the concomitant lipid dilution. Even with a high-CMC detergent, an additional centrifugation step is required to concentrate the diluted proteoliposomes. Another important drawback is that full detergent removal cannot be attained, because this would correspond to infinite dilution and, thus, the residual detergent still must be removed by other procedures.

Polystyrene Beads

Detergents with low CMCs that are not readily removed by dialysis or dilution can be removed efficiently through hydrophobic adsorption onto polystyrene bead resins such as Amberlite XAD and Bio-Beads SM-2.

The batch procedure, in which Bio-Beads SM-2 are added directly to the protein–lipid–detergent mixtures, has been demonstrated to be a powerful alternative to conventional dialysis for reconstitution trials.[19–21] Using radioactive detergents, the method has been calibrated precisely in terms of adsorptive capacity of beads and rates of detergent removal. The mechanisms underlying detergent adsorption onto beads have been analyzed, and general rules for the use of polystyrene beads have been proposed. Sufficient reproducibility can now be obtained with knowledge, experience, and careful handling, avoiding the main limitation of this strategy, which is lipid adsorption onto the beads.

This strategy is general and can be used whatever the nature of the detergent. In particular, it has been possible through this strategy to efficiently produce proteoliposomes by Triton X-100- or octaethylene glycol monododecyl ether ($C_{12}E_8$)-mediated reconstitutions. This has been an important step forward, because these low-CMC detergents, although mild for membrane proteins, were avoided in reconstitution trials because of

[18] E. Racker, B. Violand, S. O'Neal, M. Alfonzo, and J. Telford, *Biochem. Biophys. Res. Commun.* **198,** 470 (1979).
[19] D. Lévy, A. Bluzat, M. Seigneuret, and J. L. Rigaud, *Biochim. Biophys. Acta* **1025,** 179 (1990).
[20] J. L. Rigaud, D. Lévy, G. Mosser, and O. Lambert, *Eur. Biophys. J.* **27,** 305 (1998).
[21] J. L. Rigaud, G. Mosser, J. J. Lacapère, D. Lévy, A. Olofsson, and J. L. Ranck, *J. Struct. Biol.* **118,** 226 (1997).

their difficult removal by the previous conventional techniques. Another originality in the use of polystyrene beads is the possibility to master accurately the rate of detergent removal, by simply controlling the amount of beads added to the lipid–protein–detergent mixtures. Finally, another important benefit in using Bio-Beads is to remove almost all the detergent from a micellar solution, which allows the production of proteoliposomes with low ionic permeability, a crucial parameter in the study of membrane transport proteins.

From all these considerations, it appears that the use of Bio-Beads SM-2 satisfies all the criteria that make the procedure a powerful and better alternative to dialysis or dilution for proteoliposome reconstitution. This procedure is now used widely and has been demonstrated to be successful for the reconstitution of various classes of membrane proteins solubilized in all types of detergents.[4,20]

Mechanisms of Proteoliposome Formation and Efficiency of Reconstitution

Despite extensive studies and diverse applications of proteoliposomes, the mechanism of their formation has long been surprisingly ill defined. Reconstitutions from detergent micellar mixtures yielded proteoliposomes of various compositions depending on the nature of the detergent, the particular procedure used to remove it, as well as the nature of the protein and the lipid composition. Therefore, not surprisingly, each membrane protein responded differently to the various reconstitution procedures and the approach has long been entirely empirical.

In the 1990s, important knowledge of the mechanisms of liposome formation,[22] as well as understanding of the physical behavior of lipid–detergent systems,[23,24] resulted in a set of basic principles that has limited the number of experimental variables and the empirical approach of proteoliposome reconstitution.

The first basic concepts to be taken into account are those developed in the model proposed by Lasic[22] for bilayer formation by detergent depletion techniques. As detergent is removed from micellar solutions a series of micelle–micelle interactions is initiated, resulting in the formation of large mixed disklike structures. When they have grown past a critical radius, a subsequent bending of these large micelles occurs and, at a critical micelle size, the amplitude of the bending is sufficient to cause bilayer closure and

[22] D. D. Lasic, *Biochem. J.* **256,** 1 (1988).
[23] D. Lichtenberg, *Biochim. Biophys. Acta* **821,** 470 (1985).
[24] J. R. Silvius, *Annu. Rev. Biomol. Struct.* **21,** 323 (1992).

vesicle formation. Ultimately, these vesicles still undergo size transformation, as long as the level of residual detergent remains high. According to this model, the size and the morphology of the final products of a detergent-mediated reconstitution are related (1) to the size, the morphology, and the composition of the initial micelles, which are closely linked to the properties of the detergents, and (2) to the morphologies of the mixed amphiphilic structures formed during the micelle-to-lipid bilayer transition, which depend on the nature of the detergent and its rate of removal.[25–27]

Additional concepts in detergent-mediated reconstitution are related to the mechanisms that trigger protein insertion into bilayers. Two main mechanisms were initially proposed[2]: (1) detergent removal results in the simultaneous coalescence of initial lipid–detergent and lipid–detergent–protein micelles, and the protein molecules simply participate in the membrane formation process; and (2) detergent removal results in the separate dissociation of lipid–detergent and lipid–detergent–protein micelles, and the protein molecules must insert into preformed detergent-doped bilayers. The nature of the detergent used, as well as the rate of detergent removal, are critical in determining one of the mechanisms of lipid–protein association and consequently in determining the efficiency of the final proteoliposomes.[2,4,28]

To allow realistic experimental monitoring of the mechanisms by which proteins may associate with lipids during detergent-mediated reconstitutions, we have developed a strategy based on the idea that reconstitution by detergent removal from a micellar solution is the mirror image of the solubilization of liposomes by a detergent.[4,29–31] To this end, detergent was first added to preformed liposomes through the range of concentration that causes the transformation of lamellar structure into mixed micelles. This allows a ''snapshot'' of all the lipid–detergent structures that can be formed during a reconstitution process. The protein is then added at each well-defined step of the solubilization process, allowing easy determination of the optimal conditions under which a protein can associate with lipids in the presence of detergent.

Besides providing original information about the mechanisms of lipid–protein association in the presence of detergent, this ''step-by-step'' reconstitution strategy was revealed to be a powerful reconstitution procedure,

[25] P. K. Vinson, Y. Talmon, and A. Walter, *Biophys. J.* **56,** 669 (1989).
[26] A. Walter, P. K. Vinson, J. Kaplun, and Y. Talmon, *Biophys. J.* **60,** 1315 (1991).
[27] O. Lambert, D. Lévy, J. L. Ranck, G. Leblanc, and J. L. Rigaud, *Biophys. J.* **74,** 918 (1998).
[28] A. Helenius, M. Sarvas, and K. Simons, *Eur. J. Biochem.* **116,** 27 (1981).
[29] M. T. Paternostre, M. Roux, and J. L. Rigaud, *Biochemistry* **27,** 2668 (1988).
[30] D. Lévy, A. Gulik, M. Seigneuret, and J. L. Rigaud, *Biochemistry* **29,** 9480 (1990).
[31] J. L. Rigaud, M. T. Paternostre, and A. Bluzat, *Biochemistry* **27,** 2677 (1988).

more suitable than the usual methods. This strategy has produced proteoliposomes, which satisfy most of the criteria for efficient reconstitution and sustain activities comparable to those measured in the native membrane[27,32–34]

New Method for Membrane Protein Reconstitution: Step-by-Step Procedure

The new reconstitution strategy proceeds in four stages (Fig. 2): (1) preparation of large, homogeneous, and unilamellar liposomes, (2) addition of detergent to the preformed liposomes, through all the range of the solubilization process, (3) addition of solubilized protein at each well-defined step of the solubilization process, and (4) detergent removal and characterization of the reconstituted products.

Preparation of Preformed Pure Liposomes

The first stage in the reconstitution strategy is to prepare unilamellar and homogeneous preformed liposomes. Importantly, to avoid significant fusion processes on addition of subsolubilizing detergent concentrations, these liposomes may have a mean diameter exceeding 150 nm. This can be achieved using the reverse-phase evaporation method.[9,11]

A typical preparation contains 50 mg of phospholipids, usually solubilized in chloroform and dried under high vacuum. This thin lipidic film is dissolved in 3 ml of diethyl ether. Then, 1 ml of the desired aqueous buffer is added and the resulting two-phase system is sonicated, using a tip sonicator for 2 min at 4°, leading to the formation of a stable water-in-oil emulsion. The organic solvent is then removed, at room temperature, by rotary evaporation under reduced pressure (300–400 mmHg), using a nitrogen gas bleed to regulate the vacuum produced by a water aspirator. After about 15 min, a viscous gel forms that, on further organic solvent removal, collapses into a smooth suspension of large multilamellar vesicles. At this stage, an additional 2 ml of buffer is added to dilute the vesicle suspension, and evaporation (700 mmHg) proceeds for a further 30 min to remove all traces of organic solvent. The liposome suspension obtained is finally sequentially extruded through 0.4- and 0.2-μm pore size polycarbonate filters by pushing the liposomal suspension with a syringe through a filter holder.

[32] J. Cladera, J. L. Rigaud, and M. Dunach, *Eur. J. Biochem.* **243,** 798 (1997).
[33] D. Lévy, A. Gulik, A. Bluzat, and J. L. Rigaud, *Biochim. Biophys. Acta* **1107,** 283 (1992).
[34] B. Pitard, P. Richard, M. Dunach, G. Girault, and J. L. Rigaud, *Eur. J. Biochem.* **235,** 769 (1996).

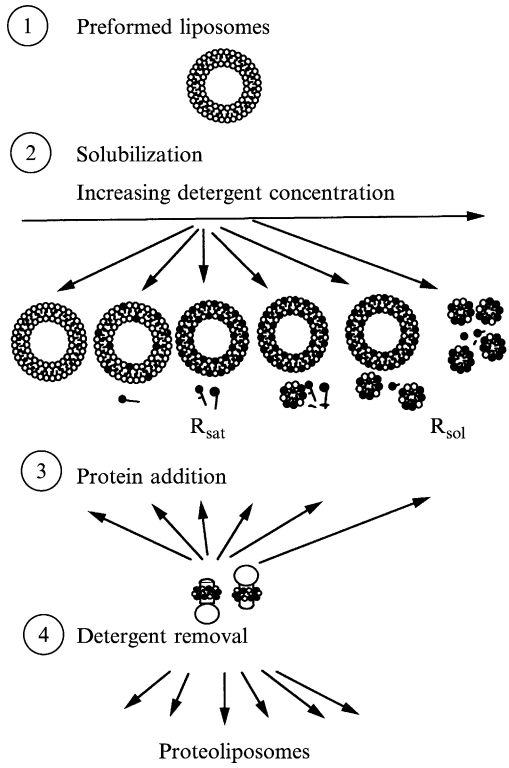

FIG. 2. The step-by-step reconstitution strategy. The standard procedure for reconstituting membrane proteins into proteoliposome is carried out in four stages: (1) preparation of large, homogeneous, and unilamellar liposomes; (2) step-by-step addition of detergent to the preformed liposomes. Some steps in the solubilization process are drawn schematically. R_{sol} corresponds to the detergent-to-lipid ratio in liposomes at the onset of solubilization, whereas R_{sat} corresponds to the detergent-to-lipid ratio in micelles when total solubilization is reached; (3) addition of the solubilized protein at each well-defined step of the solubilization process; and (4) detergent removal.

Although all our studies have been performed with liposomes produced by the reverse-phase evaporation technique, other methods that produce large unilamellar and homogeneous preformed liposomes should work as well. In particular, the Microfluidizer, a specialized apparatus commercially available from Microfluidics (Newton, MA), allows large-scale production of uniformly sized liposomes by extruding a multilamellar lipid suspension, up to 400 mg of lipid per milliliter. However, cryoelectron microscopy studies in our laboratory indicate that a significant proportion of small

liposomes is encapsulated into large liposomes. Keeping this limitation in mind, this sequential extrusion procedure can be useful when dealing with specific lipid compositions for which reverse-phase evaporation fails, because of poor lipid solubility in diethyl ether or for lipids with a high temperature transition necessitating the evaporation of organic solvent at high temperature. Other strategies, such as dialysis or gel chromatography of lipid–detergent micelles, can also be used, but care should be taken to ensure complete detergent removal.

Solubilization of Preformed Liposomes

In the second stage of the strategy, liposomes prepared by reverse-phase evaporation are diluted at the desired concentration and aliquoted to the desired volume. Solubilization is carried out by adding increasing amounts of detergent to aliquoted liposome suspensions. To monitor the solubilization process, the turbidity of phospholipid vesicle suspension (1 to 10 mg of lipid per milliliter) is measured between 400 and 700 nm as a function of detergent concentration. For lipid concentrations below 1 mg/ml, light-scattering changes are monitored with a fluorimeter set at 400 nm in both excitation and emission monochromators in order to measure the relative changes in light at 90°.

Previous studies have indicated that the solubilization process can be described by a ''three-stage'' model and quantitatively visualized through changes in turbidity of the lipid–detergent suspensions.[23,24,27,29] In stage I, the detergent partitions into the lipid bilayer liposomes until it saturates the liposomes. During this stage, detergent addition to preformed liposomes does not disrupt the liposomes and induces slight changes in turbidity. Stage II corresponds to the gradual solubilization of detergent-saturated liposomes into small lipid–detergent micelles, inducing a large decrease in turbidity. Stage III is characterized by the complete solubilization of all liposomes into lipid–detergent mixed micelles and the solution becomes optically transparent (Fig. 3).

Table I summarizes the results obtained in the solubilization process with the most commonly used detergents. From Table I, the amount of any detergent to be added to a liposome suspension to reach any step in the solubilization process can be calculated easily.

Addition of Protein

After the various detergent–phopholipid mixtures have been equilibrated for at least 1 h, the selected membrane protein is added. It is essential to add the protein as a monodisperse detergent–protein solution. This implies that, for any new membrane protein, the monodispersity of the

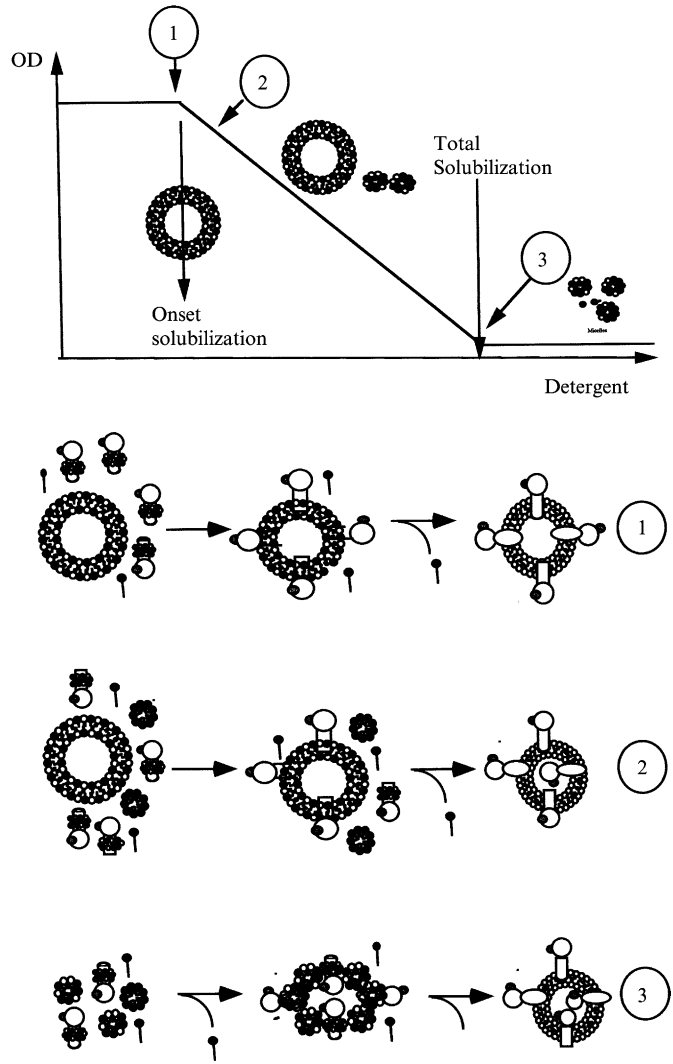

FIG. 3. Mechanisms of protein incorporation. Proteoliposomes are reconstituted according to the step-by-step strategy described in Fig. 2. *Top:* Steps in the lamellar-to-micellar transition at which optimal reconstitution has been observed, depending on the nature of the detergent. The lamellar-to-micellar transition can be analyzed qualitatively by turbidimetry as depicted schematically. *Bottom:* Depending on the nature of the detergent, proteins can either be incorporated directly into detergent-saturated liposomes at the onset of solubilization (mechanism 1), transferred from mixed micelles to detergent-saturated liposomes (mechanism 2), or participate in proteoliposome formation by micellar coalescence (mechanism 3). Note that the final orientation of the protein depends on the mechanism of association.

TABLE I
PARAMETERS DESCRIBING SOLUBILIZATION OF LIPOSOMES BY VARIOUS DETERGENTS[a]

Detergent	D_{water}		R_{sat}		R_{sol}	
	m*M*	mg/ml	mol/mol	w/w	mol/mol	w/w
Triton X-100	0.18	0.12	0.64	0.5	2.5	2.0
$C_{12}E_8$	0.20	0.11	0.66	0.45	2.2	1.5
Octylglucoside	17	4.9	1.3	0.48	3.0	1.1
Dodecylmaltoside	0.3	0.15	1	0.65	1.6	1.0
Cholate	3	1.29	0.3	0.16	0.9	0.5
CHAPS	3.15	1.94	0.4	0.31	1.04	0.8
CHAPSO	1.6	1.1	0.21	0.17	0.74	0.6

Abbreviations: CHAPS, 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; CHAPSO, 3-[(cholamidopropyl)-dimethyl-ammonio]-2-hydroxy-1-propanesulfonate.

[a] The process of solubilization can be described quantitatively by the general equation $D_{total} = D_{water} + R_{eff}$ [lipid], in which D_{total} is the concentration of the detergent to be added to reach any step in the solubilization process; [lipid] is the lipid concentration; D_{water} is the aqueous monomeric detergent concentration, that is, the CMC determined in the presence of lipids; R_{eff} is the effective detergent-to-lipid ratio in mixed lipid–detergent aggregates ($R_{eff} = R_{sat}$ at the onset of solubilization represents the detergent-to-lipid ratio in detergent-saturated liposomes; $R_{eff} = R_{sol}$ at total solubilization represents the detergent-to-lipid ratio in lipid–detergent micelles).

solubilized preparation should be checked by gel chromatography or gel electrophoresis before reconstitution.

The association of a protein with lipid–detergent mixtures has been shown to be a time-dependent process, which depends on the nature and the concentration of the detergent used for reconstitution. It is generally recommended that the protein be incubated for 1 h with the lipid–detergent mixture before detergent removal. However, this time must be decreased to about 5–10 min when dealing with detergents that can be deleterious.

Another factor is related to the concentration of the presolubilized protein. The higher this initial concentration is, the lower is the amount of detergent brought with the protein into the equilibrated lipid–detergent mixtures. A protein concentration above 1 mg/ml allows the amount of detergent brought with the protein to be minimized. As a typical example, Ca-ATPase is solubilized at 1 mg/ml in $C_{12}E_8$ at 2 mg/ml. For standard reconstitution at a lipid-to-protein ratio of 80 (w/w), 50 μl of protein solution is added to 1 ml of detergent–lipid suspension containing lipid at 4 mg/ml. Thus, the amount of detergent brought with the protein corresponds to 0.1 mg of $C_{12}E_8$, that is, to a detergent-to-lipid ratio of 0.025 (w/w).

This ratio must be compared with the detergent-to-lipid ratios between 1 and 2 (w/w) generally needed for total lipid solubilization by other detergents. A consequence of this low amount of detergent added with the protein is the possibility to perform reconstitutions of any membrane protein with any detergent different from that used for initial purification of the protein.

Detergent Removal

The last step of reconstitution is related to the removal of detergent from the equilibrated lipid–detergent–protein mixtures. Although there are various methods for detergent removal, adsorption on Bio-Beads SM-2 has been demonstrated to be the most efficient for removal of all kind of detergents and convenient for a rapid screening of reconstitution trials.[20]

Before use, Bio-Beads are washed several times in methanol followed by several more washes in distilled water and stored in water at 4°. For use in reconstitution excess water is aspirated away from Bio-Beads and a small quantity is deposited on a Kim-Wipes filter paper. After adsorption of most of the water, wet Bio-Beads are weighed quickly to prevent total drying. For detergent removal at room temperature, the standard procedure is the following: Bio-Beads are added directly to each lipid–protein–detergent solution, at a Bio-Bead-to-detergent ratio of 10 (w/w), and stirred for 1 h. A second portion of beads is then added for an additional 1 h of incubation, followed by a third addition for about 2 h to ensure complete detergent removal. For less hydrophobic detergents, such as ionic detergents or CHAPS, a fourth addition of beads is performed because of the lower adsorptive capacity of these detergents.

The most accurate method to analyze detergent removal is based on the use of radioactive detergents.[19–21] However, turbidity measurements provide a convenient way to quantitatively monitor the reconstitution process[30,34]: starting from an optically transparent micellar solution, the turbidity increases on detergent removal, reaching a plateau value at a time depending on the amount of beads added to the detergent solution. This steady state absorbance has been shown to be a good index of the end of the micelle to detergent–saturated liposome transition. However, to ensure total detergent removal from the detergent-saturated vesicles, it is recommended that an extra amount of beads be added, once the steady state has been reached.

Parameters to Be Varied

Choice of Detergent. The main lesson to be learned from past studies is that no one detergent is likely to serve equally well for the reconstitution of all membrane proteins, and the experimental approach must be kept as

broad as possible. Importantly, the choice should not be limited to the detergent used for the purification of a membrane protein. Indeed, other detergents reported deleterious when measuring the activity of a detergent-solubilized protein can be efficient, because, in reconstitution trials, phospholipids are present in excess and may have a protective effect. Thus, the optimal detergent that allows optimal protein incorporation, while avoiding its denaturation, must be found experimentally.

Regarding optimal protein incorporation, we have identified three mechanisms by which membrane proteins can associate with lipids to give proteoliposomes[4,27,32–34] (see Fig. 3). Depending on the nature of the detergent, proteins can be either incorporated directly into detergent-saturated liposomes (octylglucoside or dodecylmaltoside-mediated reconstitutions), transferred from mixed micelles to detergent-saturated liposomes (Triton X-100-mediated reconstitutions), or involved in proteoliposome formation during the micellar-to-lamellar transition (cholate, CHAPS, CHAPSC, and $C_{12}E_8$). Because reconstitution of a membrane protein into preformed liposomes ensures a unidirectional insertion of the protein in the membrane of unilamellar, homogeneous liposomes, it generally produces the most efficient proteoliposomes.[4] Thus, octylglucoside, dodecylmaltoside, and Triton X-100 are important detergents to check in reconstitution trials of any new membrane proteins. However, despite their efficiency in terms of protein incorporation, these detergents can be deleterious and/or induce protein aggregation. In this case, it is recommended that other classes of detergents also be analyzed.

As a general experimental scheme, it can be proposed that various prototypical detergents, including glycosylated detergents (octylglucoside, dodecylmaltoside), polyoxyethylenic detergents (Triton X-100 and $C_{12}E_8$), as well as ionic detergents (cholate), be tested. In preliminary trials, reconstitution should be analyzed at a few characteristic steps in the solubilization process by each of these detergents: onset of solubilization, half solubilization, and total solubilization. This allows rapid determination of the best detergent for reconstitution of any membrane protein. Once the best detergent has been determined, other parameters such as lipid composition, temperature, buffer composition, and rate of detergent removal may be optimized.

Nature of Lipids. Although all our experiments, using membrane proteins isolated from different organisms, have been performed with egg phosphatidylcholine–egg phosphatidic acid mixtures (9/1, mol/mol), preformed liposomes can be prepared from a variety of phopholipids and phospholipid mixtures for further reconstitution trials. The most commonly used phospholipids are phosphatidylcholine molecules derived from natural sources. They are often used because of their low cost relative to

other phospholipids and because of their neutral charge and chemical inertness. It is recommended that charged species such as egg phosphatidic acid or egg phosphatidylglycerol (5–20%) be added. First, these negatively charged lipids avoid liposome fusion and/or aggregation, which enables the use of a liposome preparation for 1 week. Second, full activity of many membrane proteins depends on the presence of negatively charged lipids. Cholesterol can also be included into phospholipid mixtures to provide greater stability and better impermeability of reconstituted proteoliposomes to ions and small polar molecules.

An important basic point for the preparation of well-defined liposomes is to use well-characterized lipids. Be aware that these products vary widely in purity depending on the manufacturer and even the lot number. Also important is to use the purest quality in order to avoid lipid oxidation or impurities, such as lysoderivatives, which have been shown to induce large changes in permeability of reconstituted bilayers.

When selecting a liposome composition with synthetic saturated lipids (such as dimyristoyl or dipalmitoyl derivatives), it is best to keep in mind that phospholipids form smectic mesophases that undergo a characteristic gel–liquid crystalline phase transition. This transition is a function of the chain length and it is necessary, during all the reconstitution process, to work at a temperature above the transition of the higher melting component. In the same framework, care should be taken when using non-bilayer-forming lipids (phosphatidylethanolamine) or detergent-resistant lipidic compositions (sphingomyelin, cholesterol).[35]

Lipid-to-Protein Ratio in Reconstitution Experiments. The standard lipid-to-protein ratio used in proteoliposome reconstitutions is about 80 (w/w), which is roughly equivalent to a lipid-to-protein molar ratio of 8000, considering a membrane protein of 100 kDa. At this ratio, assuming a homogeneous protein distribution, it can be calculated that a proteoliposome 200 nm in diameter contains about 25 protein molecules of 100 kDa.

For many membrane proteins, the step-by-step method allows efficient reconstitutions for lipid-to-protein ratios ranging from 800 to 10 (w/w), with the final activity increasing proportionally with the amount of protein present initially. For lipid-to-protein ratios below 10 (w/w), although all the protein could be incorporated, limitations in reconstitution efficiency are, nevertheless, related to protein aggregation, and to a drastic increase in proteoliposome permeability due to the high protein concentration in the reconstituted bilayer.

[35] R. E. Brown, *J. Cell Sci.* **111,** 1 (1998).

Rate of Detergent Removal. When reconstitutions are performed from lipid–detergent–protein micellar solutions, an additional parameter to be analyzed is the rate of detergent removal. Generally, slow detergent removal is recommended to ensure homogeneous and efficient reconstitution when starting from micellar solutions. Fast detergent removal, however, has been demonstrated to be more efficient for those membrane proteins with a high tendency for aggregation during detergent removal; for example, reconstitution of Ca-ATPase, starting from Triton X-100 or $C_{12}E_8$ micellar solutions, has been demonstrated to require fast detergent removal to avoid insertion and aggregation of the protein in a low percentage of liposomes.[33] On the other hand, fast detergent removal is required when reconstituting a protein with a detergent that can be deleterious on long exposure, as demonstrated for octylglucoside-mediated reconstitution of Ca-ATPase.[33] Finally, fast detergent removal will also be required in those detergent-mediated reconstitutions that lead to multilamellar structures, as reported for dodecylmaltoside-mediated reconstitutions.[27]

The rate of removal can be controlled easily by the regimen and the amount of beads added.[19–21] For fast detergent removal, addition at once of an amount of beads equivalent to a Bio-Beads-to-detergent ratio of 30 (w/w) is sufficient to remove all the detergent from a lipid–protein–detergent mixture in about 1 h. For slow detergent removal, successive additions, every 30 min, of small amounts of beads at Bio-Beads-to-detergent ratios of 1 to 2 (w/w) will allow the removal of the detergent in times ranging from 5 to 12 h.

When temperature must be optimized in a detergent-mediated reconstitution, it must be kept in mind that the rate of detergent adsorption onto Bio-Beads depends drastically on the temperature, doubling every 12°. This implies that either the time of detergent removal or the amount of beads must be adapted to the temperature at which the reconstitution is performed.[19,21]

Characterization of Reconstituted Proteoliposomes

Besides the need for measuring the activity of the protein, any method of membrane protein reconstitution should fulfill a number of important criteria that must be analyzed to characterize unequivocally the efficiency of the reconstitution (Fig. 4).

Functional Activity

The first parameter to analyze in order to check the efficiency of a reconstitution trial is the activity of the protein after reconstitution. Functional assays depend on the specific function of the protein under study.

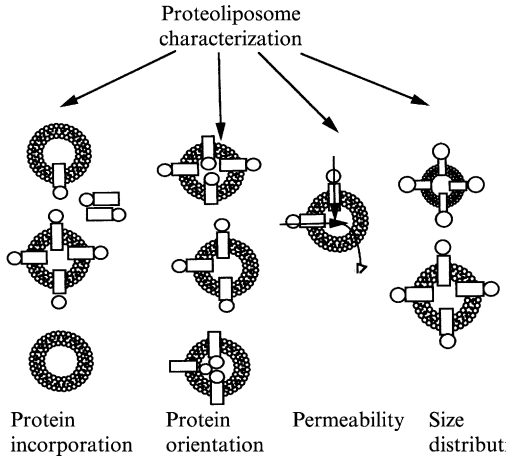

FIG. 4. Characterization of reconstituted proteoliposomes. Any method of membrane protein reconstitution should fulfill a number of important criteria. Besides the need to measure the activity of the protein, the following parameters must be analyzed: protein incorporation and protein distribution among the proteoliposomes, final protein orientation, size distribution, and permeability.

For each protein, activity measurements are set up to allow not only a measurement consuming small amounts of material, but also a rapid measurement, facilitating a rapid screening of the samples after reconstitution.

Protein Incorporation

Because nonincorporated and/or aggregated membrane proteins may still be active, it is essential to characterize a reconstitution trial by determining the amount of protein incorporated in the reconstituted proteoliposomes. The most accurate method to analyze the efficiency of membrane protein incorporation is a density gradient. The nonincorporated protein is separated easily from liposomes containing different amounts of proteins after centrifugation on a 0–30% sucrose gradient.[31–33] The reconstituted material is loaded on top of a continuous or discontinuous 0–30% sucrose gradient made in the desired buffer and centrifuged at 100,000 *g* at 4° [TLS55, 34,000 to 35,000 rpm (Beckman, Fullerton, CA) or other Beckman centrifuge with SW-type rotor and appropriate speed to achieve 100,000 *g*]. Proteoliposomes can also be subjected to discontinuous flotation gradients. In this case, the reconstituted sample is first deposited in a 30% sucrose layer. Successive layers of 20, 15, 10, 5, and 2.5% sucrose (w/w) are then deposited on top of this layer. After centrifugation the various sucrose

layers are analyzed for protein and lipid content, using colorimetric determinations or more sensitive radioactive or fluorescent labeling.

Incorporation of a membrane protein can also be monitored by freeze–fracture electron microscopy.[32,36] The presence and distribution of intramembrane particles in the fracture faces demonstrate efficiency and homogeneity of protein incorporation. Furthermore, examination of convex and concave fracture faces can reveal a significant difference in particle density related to a preferential orientation of the protein in the reconstituted membranes.

Protein Orientation

As a general approach, sidedness of membrane proteins after reconstitution can be determined from gel electrophoresis patterns before and after proteolytic treatment of the reconstituted samples.[11,32] To this end proteoliposomes (protein at 100 μg/ml) are incubated in the presence of a specific proteolytic enzyme (e.g., trypsin, chymotrypsin, Pronase, and/or papain). After the proteolytic reaction is stopped, the samples are delipidated[37] before sodium dodecyl sulfate–polyacrylamide and gel electrophoresis (SDS–PAGE). The percentage of protein orientation is determined from the ratio of the intensities of the protein band before and after proteolytic digestion, using a laser densitometer.

Functional tests, using one-sided inhibitors, must be specifically adapted to the membrane protein under study. For membrane proteins with ATPase activity, the orientation after reconstitution in the bilayer is assessed by determining the fraction of molecules accessible to one-sided inhibitors that react with the cytoplasmic domains, thus abolishing the ATPase activity [e.g., fluorescein isothiocyanate (FITC)[38] for Ca-ATPase or ouabain for Na/K-ATPase[39]]. Accordingly, the residual ATPase activity after full reaction with a one-sided inhibitor represents the fraction of ATPase molecules facing the inside of intact reconstituted vesicles. Detergent-solubilized reconstituted vesicles are generally taken as controls in which all molecules are accessible to inhibitors. For light-activated membrane proteins that function whatever the final protein orientation, measurements are simply performed in the presence or absence of a one-sided nonpermeant inhibitor (e.g., millimolar concentration of lanthanides that

[36] T. Gulik-Krzywicki, M. Seigneuret, and J. L. Rigaud, *J. Biol. Chem.* **262,** 15580 (1987).
[37] D. Wessel and U. I. Flügge, *Anal. Biochem.* **138,** 141 (1984).
[38] H. S. Young, J. L. Rigaud, J. J. Lacapère, L. G. Reddy, and D. L. Stokes, *Biophys. J.* **72,** 2545 (1997).
[39] F. Cornelius, *Biochim. Biophys. Acta* **1071,** 19 (1991).

inhibit bacteriorhodopsin proton-pumping activity when facing the carboxy terminus of the protein[40]).

Size Distribution

Various methods, already developed for liposomes, have been applied to determine not only the average size but also the size distribution of reconstituted proteoliposomes.

Light-scattering procedures, particularly laser-based quasi-elastic light-scattering methods, are popular to obtain information about the size and polydispersity of a reconstituted proteoliposome preparation.[41] These techniques are based on the time-dependent intensity fluctuations of scattered laser light due to Brownian motion of particles in solution. Analysis of fluctuation as a function of time yields lateral diffusion values that can be related to the Stokes radius of the particles. The advantage of light-scattering methods is that information can be obtained in a few minutes. However, caution must be taken because misleading results can be obtained for heterogeneous systems exhibiting bimodal or more complex size distributions.[42]

If only an approximate idea of the size range is required, gel chromatography can also be recommended, because it is a quick and convenient method for both fractionation and average size determination of proteoliposomes. Different gels can be used, depending on the mean size of the proteoliposomes. Keeping in mind that most detergent-mediated reconstitutions produce proteoliposomes less than 300 nm in diameter, Sephacryl S-1000 columns are especially appropriate for separating vesicles.[40,43]

Electron microscopy is the most precise method available to measure vesicle size, whatever the size range. Negative staining, although the simplest electron microscopic approach, must be interpreted with caution because of stain artifacts and significant distortions of large proteoliposomes that collapse on carbon-coated grids. Freeze–fracture electron microscopy is also useful for size and morphology analysis of a proteoliposome preparation.[36] However, care must be taken when measuring size distribution, because the random cleavage plane does not necessarily go through the midplane and thus can reveal a smaller diameter vesicle. Finally, cryoelectron microscopy is the best technique in characterizing the size distributions and morphology of reconstituted liposomes. It is

[40] M. Seigneuret and J. L. Rigaud, *FEBS Lett.* **228,** 79 (1988).
[41] H. Ruf, Y. Georgalis, and E. Grell, *Method Enzymol.* **172,** 364 (1986).
[42] O. Lopez, M. Cocera, E. Wehrli, J. L. Parra, and A. de la Maza, *Arch. Biochem. Biophys.* **367,** 153 (1999).
[43] J. A. Reynolds, Y. Nozaki, and C. Tanford, *Anal. Biochem.* **130,** 471 (1983).

useful in discerning changes in size and volume distributions, because this technique avoids the artifacts of staining and drying procedures and permits the observation of undistorted samples (see Chapter 29 in Volume 373[44]).

Determining the volume of the aqueous compartment of proteoliposomes is important when dealing with the activity of membrane transport proteins. Most of the methods involve measurements of radioactive or fluorescent markers trapped inside liposomes after removal of the untrapped marker by dialysis, gel chromatography, or centrifugation. It is usually also necessary to measure the amount of residual marker present outside the liposomes, because removal is often incomplete and leakage may occur during washing procedures. Fluorescence techniques have been developed that avoid the need for removal of external marker.[45] Using calcein (10^{-4} *M*) as a fluorescent marker, the addition of cobalt or copper cations (10^{-2} *M*) outside the liposomes is sufficient to quench the fluorescence of the external marker. The fraction of the total volume that is within the proteoliposomes is obtained as the fraction of the fluorescence that remains after adding cations that, when chelated by calcein, quench its fluorescence. Although a rapid and simple method, care must be taken about time-dependent aggregation of proteoliposomes containing negatively charged phospholipids.

Permeability

An important aspect in reconstitution is the residual detergent, which can alter the passive permeability of proteoliposomes. The most precise method to analyze residual permeability consists of measuring proton and counterion flux generated by an external acid pulse.[46] To this end, proteoliposomes are prepared in the presence of 200 μM pyranine, a fluorescent pH-sensitive probe ($\lambda_{ex} = 460$ nm; $\lambda_{em} = 510$ nm). After reconstitution, external pyranine can be removed by passing the proteoliposomes through a prepacked G-25 Sephadex column. The resulting pyranine-containing proteoliposomes are subjected to an external acidic pulse of 0.5 pH unit, and changes in internal fluorescence are monitored as a function of time. The changes in the rate of internal acidification, following the acidic pulse, can be related to proton and counterion permeability coefficients.

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[45] N. Oku, D. A. Kendall, and R. C. Macdonald, *Biochim. Biophys. Acta* **691,** 332 (1982).
[46] M. Seigneuret and J. L. Rigaud, *Biochemistry* **26,** 6723 (1986).

Conclusion

Reconstitution of membrane proteins into liposomes is a powerful tool that can be used to identify the mechanism of action of membrane proteins. As shown in this chapter, it appears that reconstitution is no longer ''black magic,'' and the prospects of achieving optimal proteoliposome reconstitution are obviously good when using reliable methods and systematic experimental analysis.

The future of membrane protein reconstitution appears bright in the light of the steadily expanding number of membrane proteins that have been identified in the sequencing of the genomes of different organisms. Powerful methods of overexpression of genes are now available that will allow the production of new, interesting membrane proteins. In this context, although not largely exploited up to now, the reconstitution of membrane proteins into liposomes should be an efficient strategy for the purpose of pharmaceutical, cosmetic, and chemical applications (e.g., specific targeting, drug delivery, and antigenicity or gene therapy).

[5] Reconstitution of Purified Bacterial Preprotein Translocase in Liposomes

By CHRIS VAN DER DOES, JEANINE DE KEYZER, MARTIN VAN DER LAAN, and ARNOLD J. M. DRIESSEN

Introduction

The last decade has seen a major advance in the study of bacterial protein translocation (for reviews see Refs. 1 and 2). Secretory and membrane proteins are synthesized at the ribosome with an N-terminal signal sequence or hydrophobic transmembrane segments that direct the proteins to the bacterial inner (cytoplasmic) membrane. Many inner membrane proteins are targeted to the membrane as a ribosome-bound nascent chain via the signal recognition particle (SRP) and FtsY, the SRP receptor. The targeting of most periplasmic and outer membrane proteins occurs posttranslationally as a completely synthesized polypeptide, often in association with the molecular chaperone SecB.[1] At the membrane, both

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[2] A. J. M. Driessen, E. H. Manting, and C. van der Does, *Nat. Struct. Biol.* **8,** 492 (2001).

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