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SHORT REVIEW

Strategies in the Reassembly of Membrane Proteins into Lipid Bilayer Systems and Their Functional Assay[1]

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Abstract

The various strategies available today to reassemble functional membrane proteins into model lipid bilayer systems are briefly described. Some of their virtues and weaknesses are discussed. The intimate relationships and complementarity between proteoliposomes and planar bilayers are illustrated.

Key Words: Reconstitution; membranes; membrane protein; transport; transduction; planar bilayers; spherical bilayers.

Introduction

Man has often opted for the synthetic path as one approach to understand himself and his environment. Stories such as that of Dr. Frankenstein dramatize this attitude, that is, man's struggle to reconstitute the wholeness of life in an attempt to gain control and knowledge.

Membrane biology has evolved to a point where many of the elaborate tasks performed by the membrane have been characterized and the proteins involved in these processes identified. Advances in the biochemical characterization and isolation of membrane proteins in their functional state together with their recently achieved crystallization (Tanaka *et al.*, 1980; Ozawa *et al.*, 1980, 1982; Michel and Oesterhelt, 1980; Michel *et al.*, 1980; Garavito and Rosenbush, 1980) and new developments in structural analysis (Henderson and Unwin, 1975; Fuller, 1981) have set the stage for a qualitative jump in the

[1]Abbreviations: BLM, planar black lipid membranes; PLCs, protein–lipid complexes; AChR, acetylcholine receptor; PLVs, large protein–lipid vesicles; VDAC, voltage-dependent anion channel from mitochondria.

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study of structure–function relationships in membranes. The reconstitution approach is being successfully used to unravel the structure–function relationships which will allow a closer look into the molecular mechanism of membrane transport, energy transduction, and some fundamental problems of translation of information. The reconstitution approach permits, in principle, the dissection of a complex membrane phenomenon into its molecular components with the subsequent gain of experimental control over the parameters that govern it. These parameters are usually inaccessible or very difficult to determine unambigously in the natural membrane. Furthermore, the maneuvers required to achieve the reassembly of a biologically active membrane provide an insight into the interactions between the membrane components involved in the function (Montal *et al.*, 1981).

The purpose of this short review is to briefly described various strategies available today to reassemble functional membrane proteins into model bilayer systems and to illustrate, in some instances, the virtues, weaknesses, and complementarity of the different approaches. Reconstitution implies the achievement of biological function in the reassembled model system. Thus reconstitution requires that there be reference data from the biological membrane for the functions of interest. This information has been obtained, when possible, using biochemical and electrophysiological assays performed on the biological membrane. Research on model membranes has yielded new strategies, some of which in a strict sense are not reconstitution procedures, but maneuvers allowing the incorporation of membrane proteins at different stages of purification into model bilayer systems (see Fig. 1). The latter has increased our capacity to study the molecular parameters that control and modulate biological membrane function and to acquire the necessary reference data for reconstitution. A wealth of information in the reconstitution approach lies in the careful evaluation of which and how membrane parameters (membrane state and composition) must be modulated to adjust the behavior of the model system so that it approaches that of the biological membrane.

Background

Interest in protein-free model membranes formed from lipids grew as it became clear that cells utilize the water–oil interfacial activity of some of these molecules to define anatomical boundaries (Bangham *et al.*, 1974). The introduction of planar bilayers, better known as black lipid membranes (BLMs), by Mueller *et al.* (1962) was instrumental in the characterization of the passive properties of the biological membrane and in the conceptual development of modes of membrane transport. Concepts, such as the trans-

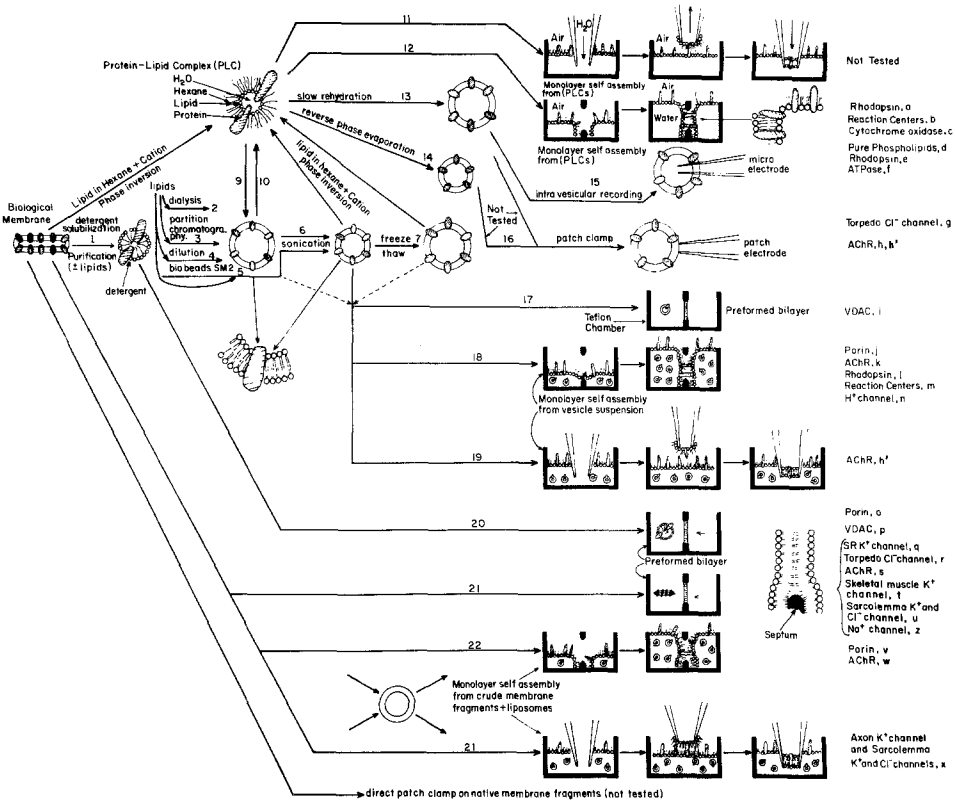

Fig. 1. Strategies in the reassembly of membrane proteins in lipid bilayer systems and their functional assay. This figure is a modified and updated version of Montal *et al.* (1981), and is a schematic representation not drawn to any precise scale. The numbers appearing on the arrows are referred to in the text when dealing with that particular strategy. The references to the examples for planar bilayer reassembly are: (a) Montal and Korenbrot (1973); Montal *et al.* (1977). (b) Schönfeld *et al.* (1979); Packham *et al.* (1980). (c) Montal (1974). (d) Antanavage *et al.* (1978). (e) Darszon *et al.* (1979c). (f) Hamamoto and Kagawa (1982). (g) Tank *et al.* (1982). (h) Tank *et al.* (1983). (h') Suárez-Isla *et al.* (1983). (i) Cohen *et al.* (1980). (j) Schindler and Rosenbush (1981). (k) Nelson *et al.* (1980). (l, m) Montal *et al.* (1981). (n) Schindler and Nelson (1982). (o) Benz *et al.* (1978). (p) Schein *et al.* (1976); Colombini (1979). (q) Miller and Racker (1979); Labarca *et al.* (1980). (r) White and Miller (1979). (s) Boheim *et al.* (1981). (t) Latorre *et al.* (1982). (u) Coronado and Latorre (1982). (v) Schindler and Rosenbush (1978). (w) Schindler and Quast (1980). (x) Coronado and Latorre (1983). (z) Krueger *et al.* (1983).

membrane channel, or pore, and the mobile carrier, received support from planar bilayer experiments with polypeptide and polyene antibiotics (Mueller and Rudin, 1969; Latorre and Alvarez, 1981). Liposomes (lyotropic smectic mesophases, i.e., spherical bilayers that trap an internal volume), another important model complementary to BLMs, began to be used after the initial characterization of aqueous suspensions of lecithin (Bangham, 1963;

Bangham *et al.*, 1965, 1974). Liposomes have proven to be a valuable model system for the study of basic biophysical and biochemical membrane phenomena, and are now being used as drug carriers (Szoka and Papahadjopoulus, 1980; Pagano and Weinstein, 1978).

In the 1960s enzymologists realized the impossibility of showing oxidative and photosynthetic phosphorylation in solutions of purified enzymes obtained from detergent-solubilized biomembranes. Even after heroic efforts, they were also unable to isolate chemical high-energy intermediates linking electron transfer to ATP synthesis (Racker, 1976; Boyer *et al.*, 1977). At this crucial stage Mitchell in his chemiosmotic hypothesis postulated a fundamental role for the membrane in energy transduction. Experimental support for the chemiosmotic hypothesis required the isolation and functional vectorial reassembly of the components involved in energy transduction into membranes. This new approach in membrane biology, in principle applicable to any membrane function, began in Racker's laboratory, where the H^+-ATPase was extracted and incorporated into liposomes which were then capable of ATP–P_i exchange and H^+ translocation (Kagawa and Racker, 1971). Cytochrome *c* oxidase in liposomes was shown to translocate protons (Hinkle *et al.*, 1972), and the $Ca^{++} \cdot Mg^+$ ATPase from skeletal sarcoplasmic reticulum displayed ATP-dependent Ca^{++} transport (Racker, 1972). Since then the field has expanded explosively; the reader is referred to several reviews dealing in detail with many aspects involved in the art of reconstitution (Racker, 1979; Montal *et al.*, 1981; Eytan, 1982; Miller, 1983; Kagawa *et al.*, 1979, 1982; Kaback, 1983).

In recent times electrophysiologists reached the important conclusion that electric excitability is mediated by integral membrane proteins capable of forming hydrophilic transmembrane channels that allow the passive diffusion of ions down their electrochemical gradients (Armstrong, 1975; Hille, 1976; Neher and Stevens, 1977). As the biochemistry of this specific group of membrane proteins advances (Agnew *et al.*, 1978; Catterall, 1982; Hartshorne *et al.*, 1982) and new ways are available to obtain reference data (see Fig. 1), the reconstitution approach becomes more attractive. This approach has already begun to deepen our knowledge about the molecular workings of biological channels (Nelson *et al.*, 1980; Schindler and Qwast, 1980; Miller, 1982; Coronado and Latorre, 1982).

Functional Reassembly of Membrane Proteins in Lipid Bilayer Systems

The expression of the full functional properties of membrane proteins involved in transport and/or energy transduction requires a membrane. The

lipid bilayer separates compartments and provides an appropriate matrix where the protein can function and orient vectorially. In addition, there may be interactions between specific lipids and the protein that modulate function (Sigrist-Nelson and Azzi, 1980; Vick *et al.*, 1981; Cortese *et al.*, 1982). Two types of model membranes have been employed most successfully in reconstitution studies, liposomes and planar bilayers.

Liposomes

When phospholipids are mechanically suspended in water, they swell and spontaneously form multilamellar liposomes, onion-like structures in which concentric closed lipid bilayers and water spaces alternate (Bangham *et al.*, 1974). Multilamellar liposomes, not very useful for reconstitution, can be transformed by sonication into smaller unilamellar liposomes of more homogeneous size (diameter 200–600 Å) (Huang, 1969; Szoka and Papahadjopoulos, 1980). Many membrane proteins have been reconstituted in liposomes; here only a few examples will be mentioned to illustrate the different reassembly strategies available. Some membrane proteins have been incorporated under special conditions into preformed liposomes (Eytan, 1982), but these procedures will not be discussed here.

Starting Material for the Formation of Protein-Containing Liposomes

Using the biological membrane as the departure point (see Fig. 1) protein-containing liposomes (proteoliposomes) can be reassembled from: (1) mixed detergent–lipid–protein micelles or from (2) protein–lipid complexes (PLCs) in organic solvents.

Mixed Detergent–Lipid–Protein Micelles

Solubilization of integral membrane proteins by formation of mixed micelles with detergent, to some degree, simulates the native situation in which the protein interacts simultaneously with both hydrophilic and hydrophobic environments (for reviews see Tanford and Reynolds, 1976; Helenius and Simons, 1975). The knowledge acquired in recent years about the properties of detergents has allowed their more rational use in the isolation of functional membrane proteins (Helenius *et al.*, 1979). Removal of detergent from mixed detergent–phospholipid–protein micelles yields proteoliposomes (Kagawa, 1972). The procedure chosen to remove detergent will be determined by the type of surfactant used and the conditions which preserve

function. Detergents difficult to dialyze because of their large aggregation number, such as Triton X-100, can be removed or exchanged by gel partition chromatography (Padan *et al.*, 1979; le Maire *et al.*, 1976) (Fig. 1, step 3) or with resins such as biobeads SM2 (Holloway, 1973) (Fig. 1, step 5). Cholate and deoxycholate, two frequently employed detergents, and octylglucoside (Baron and Thomson, 1975), which alone or combined with other detergents is giving good results (Racker *et al.*, 1975; Kasahara and Hinkle, 1976), are dialyzable. The dialysis time varies from overnight (Kagawa and Racker, 1971; Lindstrom *et al.*, 1980) to several days (Racker, 1972), depending on the conditions and the function to be reconstituted (Fig. 1, step 2). An example of the use of this method is the elegant work by Kagawa (1980) with the H^+-ATPase from thermophilic bacteria. The components of this enzyme were purified and individually reconstituted into liposomes, allowing the identification of their specific roles in ATPase function (Kagawa, 1978, 1982). In some instances, using cholate (Serranto *et al.*, 1976) and octylglucoside (Racker *et al.*, 1979), functional proteoliposomes can be formed simply by diluting the detergent (Fig. 1, step 4). Examples of the sonication procedure (Fig. 1, step 6) are the reconstitution of oxidative phosphorylation and photophosphorylation achieved by incorporating the H^+ ATPase from beef heart mitochondria together with cytochrome oxidase (Racker and Kandrach, 1973) or bacteriorhodopsin (Racker and Stoeckenius, 1974), respectively.

Although some time ago it was recognized that membrane proteins can be stabilized by the presence of excess lipid during solubilization and purification (Bruni and Racker, 1968), these steps have usually been carried out in the absence of added phospholipids. Epstein and Racker (1978) succeeded in functionally reassembling the crude acetylcholine receptor (AChR) in liposomes by using cholate and added soybean phospholipids to solubilize the AChR-containing membranes. The AchR is the best physiologically and biochemically characterized neurotransmitter receptor (Karlin, 1980). Binding of acetylcholine to it triggers the transient opening of short-lived (1–2 msec) large conductivity (~25 pS) cation-selective channels (Anholt, 1981). The realization that preservation of AChR functional integrity required the continuous presence of lipids during cholate solubilization led to the reconstitution of purified AChRs with functional cation channels into liposomes (Huganir *et al.*, 1979; Changeux *et al.*, 1979; Wu and Raftery, 1979; Lindstrom *et al.*, 1980) and planar bilayers (Schindler and Quast, 1980; Nelson *et al.*, 1980). Besides the proper stimulation and inhibition by the right pharmacological agents, the expected absolute rate of ion translocation has been observed in the reconstituted systems.

Careful studies of the protective effect of lipids with the AChR have shown that during solubilization low concentrations of lipids (20-fold molar

excess of cholate over lipid) suffice to preserve functional integrity, although at the time of detergent removal a 10-fold increase in lipid concentration was required for functional reassembly (Anholt, 1981). The presence of added phospholipids has allowed the solubilization and purification of delicate membrane proteins in their functional state (Hurt and Hauska, 1981); for example, the ability of the Na^+ channel to bind tetrodotoxin and saxitoxin has been preserved in solution (Agnew *et al.*, 1978). Goldin *et al.* (1980) used a density-shift technique, where specific transport proteins in vesicles are separated, to demonstrate veratridine-dependent Cs^+ transport by cholate + lipid solubilized brain Na^+ channels reassembled in phosphatidylcholine vesicles. Recently the functional reassembly of the purified Na^+ channel in liposomes has been reported (Weigele and Barchi, 1982; Talvenheimo *et al.*, 1982).

The procedures described so far yield mostly small unilamellar proteoliposomes with diameters that range from 300 to 1000 Å. Their size is a disadvantage for kinetic studies performed with the usual spectroscopic or radioactive exchange methods, and makes direct electric measurements with microelectrodes impossible. This situation and other factors motivated the development of procedures which allow the formation of bigger proteoliposomes with a larger trapping capacity and suitable for electric measurements with microelectrodes (Antanavage *et al.*, 1978; Darszon *et al.*, 1980). Some of these procedures will be discussed in the next section.

The freeze-thaw method of Kasahara and Hinkle (1976) used to reconstitute the glucose translocator consists of adding the membrane protein to sonicated liposomes and thereafter quickly freezing the mixture and thawing it at room temperature. Freezing and thawing induces vesicle fusion, increasing their size and making them less leaky (Pick, 1981). A small fraction of the vesicles has a diameter of up to 20–30 μm. The newly developed patch clamp technique, capable of electrically recording single-channel events on living cells by isolating a tiny piece of membrane on the tip of a polished microelectrode (Neher and Sachmann, 1976), has been applied to freeze-thawed proteoliposomes. Single-channel recording of a Cl^- channel from sarcoplasmic reticulum and from the freeze-thawed reconstituted purified AChR have been reported (Tank *et al.*, 1982, 1983; Suárez-Isla *et al.*, 1983) (see Fig. 1, step 16). The application of electrophysiological techniques to functionally reassembled proteoliposomes opens many exciting possibilities for reconstitution. Now it is feasible to compare the detailed electrical behavior of a purified channel, such as the AChR, in the various bilayer model systems and learn how their different characteristics modulate function. Medium-size liposomes (1 μm diameter) can also be formed by dialyzing mixed detergent–protein–lipid micelles in the presence of a hydrophobic surface (i.e., sephasorb) (Rögner *et al.*, 1979; Kagawa, 1982).

Protein–Lipid Complexes in Organic Solvents

Although organic solvents have been used extensively to extract small hydrophobic polypeptides from diverse membrane systems (Zahler and Niggli, 1977; Lees *et al.*, 1979), there are only few cases where function has been preserved in the organic phase (Higashi *et al.*, 1970; Sigrist *et al.*, 1977; Nelson *et al.*, 1977). In recent years a general procedure has been developed to transfer active membrane proteins into organic solvents as protein–lipid complexes (PLCs) (for a review see Montal *et al.*, 1981). These complexes in apolar solvents have been used to build several kinds of model membranes. Essentially the methodology, mostly developed with rhodopsin, the visual pigment, involves the partition of the protein from the membrane into the apolar solvent by using added phospholipids which protect the protein and enhance its transfer, and cations (usually Ca^{++}) which, in principle, neutralize the overall charge of the complex (Darszon *et al.*, 1977, 1978; Darszon, 1982). Hexane has been the solvent most frequently used. The starting material can be almost any vesicular membranous system, from biological membrane fragments (Darszon *et al.*, 1978) (Fig. 1, step 8) to proteoliposomes formed from a purified membrane protein and a single type of phospholipid (Darszon *et al.*, 1979b) (Fig. 1, step 10). The functionality of some of the membrane proteins that have been extracted as PLCs into organic solvents and reconstituted in model menbranes demonstrates that the full cycle of transferring a membrane protein from the biological membrane into organic solvents and back into a model membrane in aqueous media can be accomplished preserving the biological activity of the protein (Montal, 1974; Hwang *et al.*, 1977a; Darszon *et al.*, 1979b, 1980; Schöfeld *et al.*, 1979, 1980).

Protein–lipid complexes can be used to form small proteoliposomes after solvent evaporation and mechanical resuspension in salt media (Montal, 1974) (Fig. 1, step 9). Liposomes formed from mitochondrial PLCs have been used to estimate the number of ATPases that contain the natural ATPase inhibitor protein in its inhibitory site in intact mitochondria at various metabolic states (Darszon *et al.*, 1982; Sánchez-Bustamante *et al.*, 1982). Intermediate-size proteoliposomes (~1 μm diameter) were formed according to Szoka and Papahadjopoulos (1980) by sonicating the PLCs in the solvent with aqueous buffer followed by solvent evaporation under reduced pressure. This procedure was applied to bovine and squid rhodopsin, and the resulting proteoliposomes displayed the normal spectroscopic characteristics of these proteins (Darszon *et al.*, 1979c) (Fig. 1, step 14). Large protein–lipid vesicles (PLVs) with an average diameter of ~20–30 μm form spontaneously upon slow hydration of the protein–lipid complex residue left after solvent removal; this procedure has been applied to several membrane proteins. PLVs formed from organic extracts of rhodopsin and reaction centers from *Rhodopseudo-*

monas spheroides displayed biological activity. The PLVs were penetrated with microelectrodes and visualized by the injection of a fluorescent dye (Darszon *et al.*, 1980). In principle these proteoliposomes should be suitable for patch clamp studies. Recently Hamamoto and Kagawa (1982), using this method, were able to incorporate purified H^+-ATPase from thermophilic bacteria into PLVs and measure $\Delta\mu H^+$ by enhancement of fluorescence of anilinonaphthalene sulfonate on addition of ATP-Mg^{++}. These vesicles were also impaled with microelectrodes.

Planar Bilayers

Among the main attractions of planar bilayers are their suitability for high-resolution electrical measurements and access to and control over the environment on both sides of the membrane. Their main application in reconstitution work has been in the study of membrane functions associated with the translocation of charges across the lipid bilayer, that is, channels and pumps. The electric current going through a single channel is of the order of 10^7 charges per second, equivalent to 1.6 pA, a quantity easily detectable with commercially available amplifiers. On the other hand, the study of electrogenic pumps is more difficult since the typical turnover of a single pump is $\cong 100\ sec^{-1}$, equivalent to 1.6×10^{-17} A. Thus to measure the current that goes through a single channel, around 10^5 pump molecules must be incorporated into the planar bilayer (see Miller, 1983, for further discussion). Using special methods it has been possible to achieve larger pump currents (Drachev *et al.*, 1974; Herrman and Rayfield, 1978; Bamberg *et al.*, 1979; Korenbrot and Hwang, 1980). Radioactive tracer experiments can be performed in planar bilayer reconstitution studies, although extremely stable membranes, luck, and an extraordinary patience are required (Nickson and Jones, 1982).

Membrane proteins can be reassembled into planar bilayers essentially by two procedures (for reviews see Montal *et al.*, 1981; Miller, 1983; see right-hand side of Fig. 1 for examples).

The first procedure is a sequential one: a lipid planar bilayer is first formed either by the Mueller–Rudin technique (containing solvent) or by the apposition of two lipid monolayers (Montal and Mueller, 1972), and subsequently the protein is incorporated. Two approaches have been used for protein incorporation: (a) fusion of protein-containing vesicles with the preformed bilayer (Fig. 1, step 17, 21) and (b) insertion of detergent-solubilized protein (Fig. 1, step 20). With the exception of VDAC this last approach has failed with channels from higher organisms (Montal, 1976). The fusion approach has been the most widely used and best studied (Miller and Racker, 1976; reviewed by Miller, 1983). The vesicles added to the

subphase for diffusion can be proteoliposomes formed from purified proteins (Fig. 1, step 17) or crude membrane fragments (Fig. 1, step 21). The conditions that favor fusion are Ca^{++}, acidic lipids, phosphatidylethanolamine, and osmotic gradients (Miller and Racker, 1976; Cohen *et al.*, 1980; Zimmerberg *et al.*, 1980). When more than one channel is present in the membrane fragments used for fusion, it is not clear why in some cases only one type of conductance is preferentially observed in planar bilayers (Miller, 1978; White and Miller, 1979; Latorre *et al.*, 1982). Compared to the total number of vesicles, those that fuse are very few; therefore it is possible that only a minor fraction of the vesicle population selectively fuses without statistically sampling the channels present. Vesicle–bilayer fusion can also be induced by phase transitions, and channels can be incorporated by this less well characterized procedure (Boheim *et al.*, 1980, 1981).

The second procedure is a simultaneous one: protein and lipid are reassembled together by the apposition of two monolayers. The self-assembly of the mixed monolayers required for this procedure can be achieved by two methods: (a) in the first, protein–lipid complexes in volatile organic solvents are spread into monolayers at air–water interfaces (Montal, 1974; Montal *et al.*, 1981). The protein is treated with organic solvent, and thus one has to prove that under defined extraction conditions the protein remains functional (for example, see Darszon *et al.*, 1979b; Schöenfeld *et al.*, 1980); (b) in the second method monolayers spontaneously form at the air–water interface of vesicle suspensions (Schindler and Rosenbush, 1978; Schindler, 1979). The monolayers are generated when either crude membranes mixed with liposomes (Fig. 1, step 22) or proteoliposomes formed with a purified protein (Fig. 1, step 18) interact with the air–water interface. This strategy permits the correlation of functional activity in the planar bilayer with that found in the biological membrane or in reconstituted proteoliposomes.

The monolayer is certainly not the natural environment of a membrane protein, and protein conformation may be distorted. Although it has been shown that rhodopsin, bacteriorhodopsin, and cytochrome oxidase remain functional after monolayer self-assembly, some structural observations indicate that the protein is not in a monolayer configuration but in a bilayer (Korenbrot and Pramik, 1977; Hwang *et al.*, 1977a, b; Montal, 1974). More information is required about the structure of the region in the interface where the protein is located to better understand the simultaneous reassembly method.

Recently it has been possible to form lipid bilayers from a monolayer at an air–water interface on the tip of microelectrode patch-pipettes by successive removal of the pipette from the water and reimmersion through the interface (Wilmsen *et al.*, 1982; Hanke *et al.*, 1983; Coronado and Latorre, 1983). This strategy allows the functional reassembly of membrane proteins

into lipid bilayers (see Fig. 1, steps 19 and 23 for examples). It has the additional advantage over conventional planar bilayers of allowing measurements with higher signal-to-noise ratio and faster time resolution (Suárez-Isla *et al.*, 1983).

Concluding Remarks

The reconstitution of membrane proteins into liposomes is coming to maturity. It is now possible to study reconstituted proteoliposomes using a wide range of biochemical and biophysical assays, from the usual spectroscopic and radioactive exchange methods to direct electrical recordings. This favorable situation has increased our capacity to derive membrane structure–function relationships. There are still important problems such as the control of lipid and protein asymmetry (Brethes *et al.*, 1981), lipid–protein stoichiometry, and proteoliposome size, whose solution will bring to light basic parameters that govern lipid–protein interactions and membrane assembly.

Although reconstitution of membrane proteins into planar bilayers is at a more primitive stage, it has already begun to yield new information at the molecular level about well-known channels. The successful incorporation of channels from biological membrane fragments into planar bilayers has provided valuable new ways to obtain background information for reconstitution. The electric measurements performed on planar bilayers are very sensitive and therefore very prone to artifacts; however, as the background information about transport proteins increases and better ways to analyze electric activity are developed, the task of dissociating artifacts from relevant biological events becomes less difficult.

Figure 1 illustrates the intimate relationships and complementarity between proteoliposomes and planar bilayers. The diversity in the available membrane reassembly strategies promises exciting advances in this area.

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