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Review

Use of detergents in two-dimensional crystallization of membrane proteins

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Abstract

Structure determination at high resolution is actually a difficult challenge for membrane proteins and the number of membrane proteins that have been crystallized is still small and far behind that of soluble proteins. Because of their amphiphilic character, membrane proteins need to be isolated, purified and crystallized in detergent solutions. This makes it difficult to grow the well-ordered three-dimensional crystals that are required for high resolution structure analysis by X-ray crystallography. In this difficult context, growing crystals confined to two dimensions (2D crystals) and their structural analysis by electron crystallography has opened a new way to solve the structure of membrane proteins. However, 2D crystallization is one of the major bottlenecks in the structural studies of membrane proteins. Advances in our understanding of the interaction between proteins, lipids and detergents as well as development and improvement of new strategies will facilitate the success rate of 2D crystallization. This review deals with the various available strategies for obtaining 2D crystals from detergent-solubilized intrinsic membrane proteins. It gives an overview of the methods that have been applied and gives details and suggestions of the physical processes leading to the formation of the ordered arrays which may be of help for getting more proteins crystallized in a form suitable for high resolution structural analysis by electron crystallography.

Keywords: Membrane protein; 2D crystal; Detergent; Electron crystallography; 2D crystallogenesis; Lipid layer

1. Introduction

About one third of the sequenced genes in yeast are predicted to code for membrane proteins and an even larger fraction code for membrane associated proteins. This large fraction reflects the importance of membrane proteins which cover a wide spectrum of fundamental biological processes. The function, sequence and topology of many of these membrane proteins have been extensively studied. However, despite the important information obtained from biochemical, biophysical and molecular biology strategies, our understanding of membrane phenomena has been severely hindered by a dearth of structural information. Indeed, despite many efforts, only few distinct transmembrane proteins have been solved to atomic resolution. Thus, elucidation of the three-di-

Abbreviations: $C_{12}E_8$, *n*-dodecyl-octaethylene-glycol monoether; dodecylmaltoside, 1-*o*,*n*-dodecyl-β-D-maltoside; octylglucoside, *n*-octyl-β-D-glucopyranoside; octylthioglucoside, *n*-octyl-β-D-thioglucopyranoside; heptylthioglucoside, *n*-heptyl-β-D-thioglucopyranoside; hecameg, 6-*o*-(*N*-heptyl-carbamoyl)-methyl-α-D-glucopyranoside; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio-2-hydroxy-1-propanesulfonate; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; octylPOE, octyl polyoxyethylene; cmc, critical micelle concentration

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mensional structure of membrane proteins remains a major challenge. Such knowledge is not only important for academic reasons since it permits rational structure–function relationships to be established at a molecular level, but will have considerable impact on the ability to design potent and selective drugs interacting with membrane proteins that are of therapeutic interest.

Membrane proteins are generally considered as the most difficult to crystallize, mainly due to their amphiphilic character which implies the use of detergent for their solubilization and their purification [1,2]. Success in growing three-dimensional crystals in detergent is relatively rare, probably because of the difficulty in producing and/or maintaining a crystal lattice through the sole interactions between extramembrane domains of the proteins, the hydrophobic domains being shielded by the detergent micelles [3,4]. The bottleneck of 3D crystallization has been addressed by Michel's group who has introduced a novel approach in which monoclonal antibody fragments are bound to the membrane complex with the idea of extending the hydrophilic domains of membrane proteins [5]. Therefore, because of the tremendous difficulty in 3D crystallization, less than 25 original structures of membrane proteins have been solved by X-ray crystallography, a number which lags far behind that of soluble proteins.

To help with this difficult problem, reconstitution of membrane proteins into artificial membranes to form crystals confined to two dimensions (2D crystals) has opened a new way to solve their structure into a native-like environment. Indeed, electron crystallography has developed for the past few years to that point that it now represents an excellent alternative and a complementary discipline to X-ray crystallography in membrane proteins structural biology. Atomic models for bacteriorhodopsin [6] and light-harvesting complex [7] were derived from electron crystallography of 2D crystals and high resolution studies of porin [8] also looked promising until they were abandoned upon publication of the X-ray structure. Besides these examples, many other 2D crystals have led to resolutions which allowed the secondary structure to be seen and work towards determining their structures to high resolution are making good progress [9–21]. Although it is difficult to list all the information published since the last 10 years of extensive research, a number of excellent reviews are available concerning 2D crystal formation of different specific classes of membrane proteins and their analysis by electron crystallography [22–26].

However, one of the major bottlenecks in reaching structural information through this strategy relies on the production of well-ordered 2D crystals. Besides a few membrane proteins that occur as regular arrays in native membranes (bacteriorhodopsin [6], connexin [20]) and other 2D crystals produced by rearrangement within the membrane (Ca-ATPase [18], acetylcholine receptor [12], rhodopsin [16]), most of the 2D crystals have been produced from purified detergent-solubilized proteins. The self-assembling process leading to densely packed vesicles and 2D crystals depends upon a large matrix of variables. In addition to the composition of the solution (salt, pH, additives such as glycerol) and temperature, variables of primary importance are related to protein–protein, lipid–protein, detergent–protein and detergent–lipid interactions. However, as yet as the number of well-studied examples of crystallogenesis is very low, many parameters are poorly understood and the approach is rather empirical, limiting the number and/or the quality of 2D crystals produced.

This review deals with the different strategies commonly used to produce 2D crystals of membrane proteins starting from detergent-solubilized purified material. It gives an overview of the methods that have been applied with a peculiar attention to very recent experimental approaches, that have allowed promising perspectives to be foreseen in producing more 2D crystals of membrane proteins amenable to structural analysis. In the present review, we will also focus on and discuss recent contributions that have led to new insights into the mechanisms of detergent action during the formation of 2D crystals. Although far from generalization, some guidelines appear to emerge that will promote the increase of an integrated approach to this field that has often seemed more like art than like science.

2. 2D crystallization by controlled solubilization of native membranes

Some membrane proteins have a natural propen-

sity to form regular 2D arrays within the native membranes. The best example is bacteriorhodopsin which forms highly ordered 2D crystals in the plasma membrane of *Halobacterium salinarium*. Such 2D crystals were used for the first structure determination of a membrane protein at high resolution [6]. Another example of highly ordered natural 2D crystals is the acetylcholine receptor which rearranges spontaneously within membrane vesicles isolated from the electric organ of the *Torpedo* ray [12]. Unfortunately, very few other membrane proteins form similarly well-ordered 2D arrays in vivo [27,28]. However, it has to be stressed that some specialized biological membranes which contain high levels of a few different proteins with a high packing density have been useful for 2D crystallization of membrane proteins. In these particular cases, ordered arrays result from rearrangements of the protein within the membranes following induction by specific agents (e.g. vanadate-induced crystallization of the Ca-ATPase [18]) or treatment of isolated membranes by detergents.

The effect of detergents has been related to an in vivo solubilizing effect leading to removal of lipids and/or other proteins from the isolated membranes. For example, it has been reported that extraction of cytochrome oxidase from beef heart mitochondria with Triton X-100 and Triton X-114 resulted in the removal of other major membrane proteins as well as some lipids which in turn resulted in the crystallization of this membrane protein [29,30]. In the same framework, another 2D crystal form of cytochrome oxidase was produced after treatment of the isolated membranes with deoxycholate instead of Triton [31]. As another example, isolation of gap junctions with deoxycholate and Lubrol led to the formation of hexagonal arrays of connexins due to the removal of some lipids by these detergents [32]. The same effect of controlled solubilization of detergents has also been reported after treatment of rod disk membranes by the non-ionic detergent Tween 80 leading to 2D arrays of rhodopsin [33].

Whatever, such examples of biological membranes enriched in one type of membrane protein are very few and most 2D crystals have to be produced from isolated, purified membrane proteins.

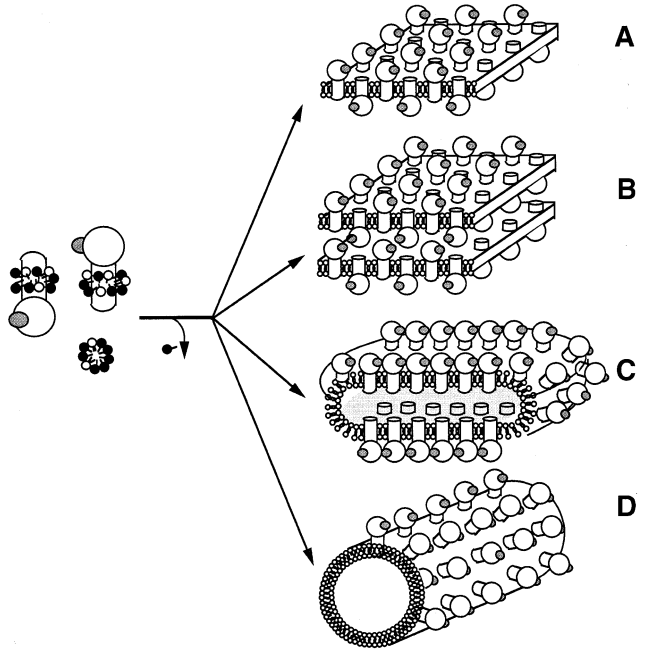

Fig. 1. 2D crystallization of membrane proteins by detergent removal. Following detergent removal from a lipid–protein–detergent micellar solution, different 2D crystals can be produced. (A) Planar 2D crystals; (B) stack of planar 2D crystals; (C) vesicular crystals; (D) tubular crystals.

3. 2D crystallization of isolated membrane proteins by detergent removal

To date, the most employed strategy for 2D crystallization relies on the general method of detergent-mediated reconstitution of membrane proteins into liposomes but at low lipid/protein ratios [34]. The strategy consists of start with the purified protein and the suitable combination of lipids, both solubilized in detergent. Next the detergent is removed from these lipid–detergent and lipid–protein–detergent micellar solutions, resulting in the progressive formation of lipid bilayers in which the proteins incorporate and eventually crystallize (Fig. 1). Several types of 2D crystals, which all contain a continuous lipid bilayer in which proteins have been incorporated, can be produced: vesicular crystals; tubular crystals in which reconstituted proteins are helically ordered on the surface of a cylinder; planar crystalline sheets which in some instances can lead to thin 3D crystals upon the stacking of the 2D arrays.

Whereas the intermolecular contacts in 3D crystals involve specifically the extra-membrane hydrophilic

Table 1
Use of detergents in 2D crystallization of membrane proteins

Detergent	Protein	Strategy	Ref.
Triton X-100	outer membrane phospholipase A	dialysis	[85]
	glutathione transferase	dialysis	[61]
	cytochrome bo ubiquinol oxidase	dialysis	[89]
	mechano-sensitive channel	bio-beads	[47]
	cytochrome bc1	bio-beads	[49]
		dialysis	[87]
	ubiquinone: cyt c reductase	bio-beads	[86]
	PSI	bio-beads	[91]
	cytochrome reductase	dialysis	[100]
C12E8	band 3	dialysis	[99]
	Ca-ATPase	bio-beads	[42]
C8E4	rhodopsin	dialysis	[93]
Dodecylmaltoside	lactose permease	dialysis	[84]
	Na^+/H^+ antiporter	dialysis	[21]
	melibiose permease	bio-beads	[39]
	LH2	dialysis	[97]
	KcsA K^+ channel	dialysis	[83]
Decylmaltoside	cytochrome oxidase	dialysis	[94]
Octylglucoside	aquaporin	dialysis	[13–15]
	LH1 *Rhodospirillum rubrum*	dialysis	[79]
	LH2 *Rhodobacter sphaeroides*	dialysis	[96]
	LH2 *Rhodovulum sulfidophilum*	dialysis	[77,78]
	rhodopsin	dialysis	[92]
Hecameg	cytochrome b6f	bio-beads	[45,46]
Octylthioglucoside	PSI	dialysis	[80,81]
	PSI	bio-beads	np
	PSII	dilution	[101]
Heptylthioglucoside	PSII	bio-beads	[48]
	PSII	dialysis	[98,102]
Hexadecyl PC	LH1 *R. sphaeroides*	dialysis	[96]
	LH1 reaction center	dialysis	[96]
OctylPOE	porin OmpF	dialysis	[105]
	porin OmpF	dilution	[50]
	band 3	dialysis	[90]
	maltoporin	dialysis	[104]
LDAO	rhodopsin	dialysis	[82]
	LH2 *R. gelatinosus*	bio-beads	np
	reaction center	dialysis	[95]
	reaction center	bio-beads	np
LDAO+OTG	FhuA	bio-beads	[44]
	LH2	bio-beads	np
SDS	porin PhoE	dialysis	[103]
CHAPS	mannitol transporter	dialysis	[88]

domains of the detergent-solubilized proteins, the intermolecular contacts in membrane-embedded 2D crystals involve also the intrinsic hydrophobic domains of the proteins. Thus as opposed to proteoliposome reconstitution at high lipid to protein ratios (in the range of 1 protein per 2000–10 000 lipid molecules), 2D crystallization at low lipid to protein ratios (in the range of 1 protein per 10–50 lipid molecules), involves more specifically protein–protein and lipid–protein interactions. To favor protein arrangements in 2D crystals, these interactions should be considered in analogy to solvatation interactions in crystallization of soluble proteins. From such considerations, it is not surprising that the nature of both

protein and lipid as well as the lipid to protein ratios are generally the most essential parameters in the 2D crystallization of membrane proteins. However, it is also obvious that the nature of the detergent and the particular procedure used for its removal are also critical determinants of success. Indeed, besides the need to preserve the activity of the protein, the nature of the detergent controls the sequences of events during reconstitution and has a role in determining the morphology and the size of the reconstituted samples as well as in determining the incorporation of the protein and its further crystallization.

3.1. Choice of the detergent

From the literature, the relevance of the nature of the detergent in 2D crystallization trials is not clear since systematic studies as a function of detergent composition have not been performed and/or reported. Thus, to day, no general recommendation can be made on which detergent or detergent mixture is most suitable for any particular membrane protein. The choice of detergent is totally empirical and in fact relies mainly on the detergent chosen by the biologist to isolate and purify the membrane protein of interest. Indeed, it is assumed that the formation of coherent crystalline patches requires, at least, the purified protein to be solubilized in a detergent under an active form and as a unique oligomeric species.

The known examples of successful crystallization are listed in Table 1 together with the detergent conditions. At the early beginning of 2D crystallization it was thought that long-chained detergents were a prerequisite for growing coherent 2D crystals: this was related to the idea that 2D crystallization should be a long process to allow crystal growth and thus should require detergent with low cmc, i.e. detergent that could be removed very slowly by dialysis. But for many membrane proteins, detergents with very high cmc's, such as glycosylated detergents, were found to be the only suitable detergents. It can be foreseen from Table 1 that most of the detergents classically used in membrane protein biochemistry have been successful in 2D crystallization trials, whatever the cmc, the charge, the hydrophobic/hydrophilic balance or the polar head structure and the hydrophobic chain composition. However, in most cases, detergents used were non-ionic mild detergents, and very few exceptions were ionic detergents. This could be related not to a crystallization difficulty but to the fact that mild detergent with long alkyl chains and bulky head groups are advantageous to purify and maintain the activity of the protein, as compared to ionic harsher detergents.

Therefore, the main lesson to be learned from past 2D crystallization studies is that no detergent is likely to serve equally well for the reconstitution of all membrane proteins and the experimental approach has to be kept as broad as possible. The optimal combination of protein and detergent has to be found experimentally and should be tested on a larger scale through comparative studies on different classes of membrane proteins. Taking advantage of the possibility to exchange detergents, other detergents than the one used for solubilization and purification should be analyzed in comparative 2D crystallization studies of one protein in different detergents. For example, it has been shown that a detergent reported deleterious when measuring the activity of a solubilized protein can be very efficient in reconstitution trials since addition of lipids and rapid detergent removal can protect the protein against denaturation [35]. Finally, new detergents should be also analyzed, including for example short-chained phospholipids which have been shown successful in recent crystallization trials [11,36].

3.2. Different strategies for detergent removal

As stated above, besides the nature of the detergent, the particular way to remove it is a key parameter in 2D crystallization trials.

Dialysis is the most widely used technique in 2D crystallization trials. Due to the necessity to scale down the amount of material, microdialysis devices have been used in the form of small compartments (50–100 μl) dialyzed against large buffer volumes. The dialysis method has been successfully applied to many membrane proteins, but could be not well suited for detergents with low cmc's which require a long time for dialysis. Indeed, a long time exposure could not be compatible with those membrane proteins of poor stability in detergent. Using a more sophisticated device flow-through dialysis cell can be advantageous as the rate of detergent removal

can be carefully controlled and dialysis time largely decreased [37].

The method of detergent removal by hydrophobic adsorption onto polystyrene beads, previously shown very efficient for proteoliposome reconstitution [34,38], has been recently demonstrated to be a powerful alternative to conventional dialysis for 2D crystallization trials [39]. In particular, it allows to remove in relatively short times any kind of detergent, which is an important advantage for detergent with low cmc. Using radioactive detergents, the method has been precisely calibrated 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 (for a review see [40]). Sufficient reproducibility can be now obtained with knowledge, experience and careful handling. An important benefit of this new strategy has been to produce new 2D crystals of several membrane proteins solubilized in various detergents [40–49] and, importantly, some of these 2D crystals have been useful for high resolution structural analysis [44,46,48,49]. Another important advantage and originality in the use of polystyrene beads was the possibility to vary and control the rate of detergent removal, by simply controlling the amount of beads. This has been demonstrated to allow a possible identification and control of parameters critical for the formation of proteoliposomes [34] and 2D crystals ([40,42]; see below). The drawback of this technique is the difficulty in quantification of very small amount of beads that would be needed for very slow detergent removal rates, comparable to those encountered in dialysis trials.

Although scarcely used, dilution of micellar solutions has been shown efficient to produce 2D crystals of membrane proteins [50]. The principle of this strategy is to dilute a lipid–protein–detergent micellar solution to lower the detergent concentration below its cmc. This strategy has two main advantages that are related to the relatively short times for decreasing detergent concentrations and to the possibility to vary the rate of dilution by progressive addition of the dilution buffer. However, since the protein is also diluted, this strategy requires high protein concentration and whatever is only useful for high cmc detergent. Another important drawback could be related to the residual detergent which should be, in any case, removed by other procedures.

From all these considerations, it appears that all the strategies used for detergent removal have their own advantages and their own inconvenient in 2D crystallization trials and thus, no specific recommendation can be done. An interesting approach would be to combine these strategies, in order to control and vary the rate of detergent removal at the desired step of the reconstitution process. Finally, another parameter that should be analyzed is the residual detergent: except with the beads strategy such a parameter has never been quantified although it could be of primary importance for crystal growth after reconstitution.

3.3. *Proposed mechanisms for 2D crystal formation upon detergent removal*

As most of the researchers have been mainly interested in the development of crystallization methods that work for their protein and not in the physicochemical parameters involved in the preparation procedures, very few detailed 2D crystallogenesis studies have been reported. Whatever, it is now admitted that some concepts drawn from detailed analysis of the mechanisms of liposome and proteoliposome detergent-mediated reconstitution should apply in 2D crystallization trials.

3.3.1. *Bilayer formation by detergent depletion techniques*

In a first place, the concepts to be taken into account are those developed in the model proposed by Lasic [51] for bilayer formation by detergent depletion techniques (Fig. 2). The basic concepts are, as detergent is removed from micellar solutions, a series of micelle–micelle interactions initiated to minimize the unfavorable energy resulting from consequent exposure of lipid and/or protein hydrophobic regions to the aqueous medium. This results in large mixed disk-like structures whose edges are coated with detergent. When they have grown past a critical radius, a subsequent bending of large micelles to form curved micelles occurs. At a critical micelle size, the amplitude of the bending is sufficient to cause bilayer closure and thus vesicle formation. Ultimately, these initially formed vesicles still undergo size transforma-

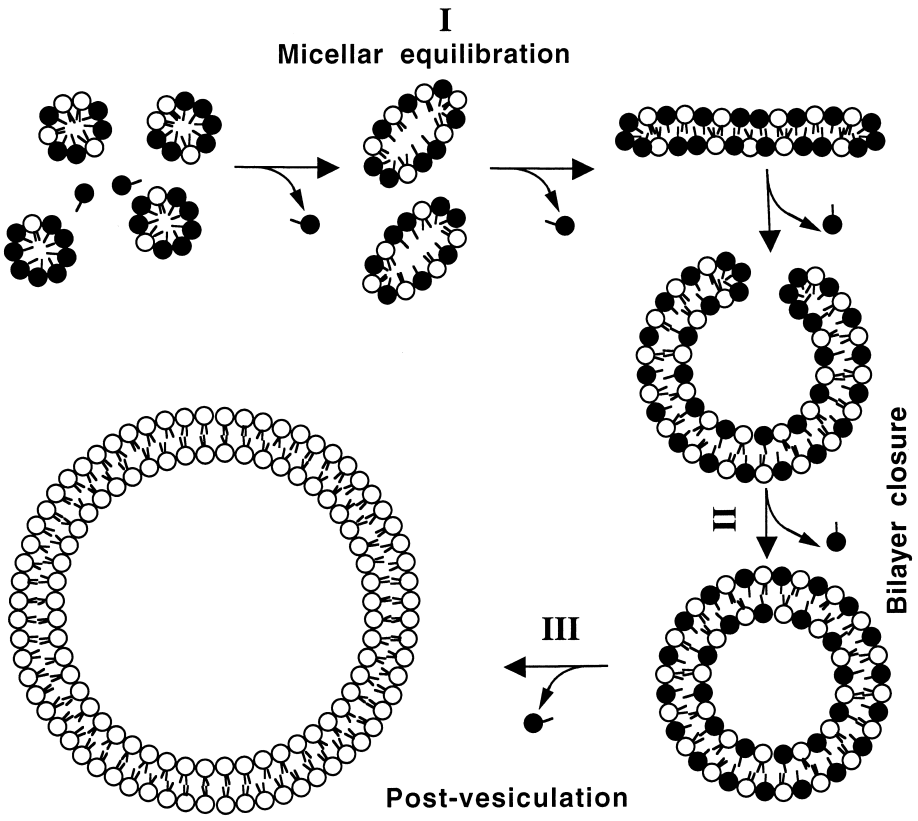

Fig. 2. Schematic representation of bilayer formation by detergent depletion techniques. Three steps occur in the overall process: micellar equilibration (I), vesiculation (II) and post-vesiculation size growth (III). Detergent removal from lipid–detergent micelles causes the transformation of small micelles to larger ones (I), which bend upon further detergent removal to form curved mixed micelles. Subsequently, detergent-saturated vesicles are formed (II) which undergo a size growth through fusion and lipid exchange mechanisms (III), leading ultimately to large unilamellar liposomes.

tion process as long as the level of residual detergent remains high. Many results are consistent with the above simplest model scheme for liposome and proteoliposome formation [34,52,53]. In addition, there is a general agreement that the three steps in the overall process of reconstitution (micellar equilibration, vesiculation and post-vesiculation size growth) are in many respects the reverse of the solubilization process (detergent incorporation, lamellar to micellar transformation, decrease in the size of the lipid–detergent micelles) [52–55].

According to the model proposed by Lasic, the size and the morphology of the final products of any detergent-mediated reconstitution are related: (i) To the size, the morphology and the composition of the initial micelles which are closely linked to the properties of the detergents [1]. It has been shown that the sizes of reconstituted liposomes or proteoliposomes are strictly dependent upon the nature of the detergent, likely due to the large differences in the size and composition of the initial mixed micelles ([34,40]. (ii) To the morphology of the mixed amphiphilic structures which coexist during the 'vesiculation' stage and which can be large bilayered aggregates, rod-like micelles or long extended micelles depending upon the nature of the detergent [54,56–58]. For example a cryo-electron microscopy study of dodecylmaltoside-mediated reconstitutions [58] has revealed the existence, in the early stage of detergent removal, of a specific very viscous 'gel-like' phase consisting of very long thread-like micelles. The importance of this 'gel-like' phase was demonstrated to drastically influence the morphology of reconstituted products, leading in dodecylmaltoside-mediated reconstitutions to large multilamellar vesicles as opposed to smaller and unilamellar liposomes with many other detergent-mediated reconstitutions.

We believe that these findings are also important

in the context of 2D crystallization because it correlates with observations that morphology of 2D crystals are dependent upon the nature of the detergent. For example, size and shape of porin OmpF lattices were shown to drastically depend on the detergent used [37]. In the same framework, we have analyzed the structures formed during 2D crystallization trials of different membrane proteins, including bacteriorhodopsin, FhuA, LH2 and photosystem I. Keeping constant all other conditions, the size and morphology of the final products were shown very dependent upon the nature of the detergent initially present. Interestingly, octylthioglucoside was reported to significantly increase the size of reconstituted 2D crystals ([44] and manuscript in preparation). These data tend to suggest a specific effect of this detergent in increasing the size of reconstituted 2D crystals, probably related to some specific physico-chemical properties of its thiol group [59].

From another point of view, according to the concepts developed by Lasic, since micelle fusion and post-vesiculation growth are not instantaneous processes, the rate of detergent removal is expected to be a key parameter in determining the size, the morphology and the composition of the final products. In this context, fast detergent removal has been demonstrated to produce much smaller liposomes than slow detergent removal [38,55]. The importance of these kinetic factors was also demonstrated in 2D crystallization of porin and much larger vesicular crystals were produced upon slow dilution of the detergent octylPOE as compared to fast dilution [50]. Interestingly, recent systematic crystallogenesis studies of the sarcoplasmic reticulum Ca-ATPase definitely demonstrated the importance of these concepts in 2D crystallization experiments [39,42,43]. Indeed, depending upon the rate of detergent removal, 2D crystals with different sizes and morphologies could be produced. Starting from Ca^{2+}-ATPase and lipids solubilized in $C_{12}E_8$, the detergent was removed with controlled amounts of bio-beads. When the detergent was slowly removed by adding successive small amounts of beads, flat multilamellar crystals of 1–5 μm in diameter were produced. These very large crystals were composed of a stack of two-dimensional crystalline lamellae. When the detergent was rapidly removed by adding an excess of beads, much smaller tubular 2D crystals were produced, about 0.2–0.5 μm in length and 0.2 μm in diameter. These last 2D crystals appeared as unilamellar crystals, indicating that the stacking observed upon slow detergent removal had no time to develop during fast detergent removal. In the same framework, using bio-beads as detergent removing agent, different types of 2D crystals of the melibiose permease were produced depending upon the rates of detergent removal [39,43]: thin tubular crystals, 40 nm width, were formed upon fast dodecylmaltoside removal, while vesicular structures, 1 μm diameter, were observed upon slow detergent removal.

3.3.2. *Mechanisms of protein incorporation and crystallization*

In a second place, the other important concepts in detergent-mediated reconstitution are related to the mechanisms that trigger protein insertion into bilayers during the micelle to lipid bilayer transition. From a throughout study of detergent-mediated reconstitution at high lipid to protein ratios, two main mechanisms for protein insertion have been proposed [34]: (1) detergent removal results in the simultaneous coalescence of mixed lipid–detergent and lipid–detergent–protein micelles and the protein simply participates in the membrane formation process; (2) detergent removal results in the separate dissociation of lipid–detergent and lipid–detergent–protein micelles and the protein molecules have to insert into pre-formed detergent-doped bilayers. The nature of the detergent used as well as the rate of detergent removal are critical in determining one of the mechanism of lipid–protein association but are rather insensitive to the nature of the protein. However, it has to be stressed that these mechanisms of protein insertion have been evidenced at very high lipid to protein ratios and the question whether they apply in 2D crystallization trials, i.e. at very low lipid to protein ratios, is still open.

Despite very few experimental evidences, three mechanisms for detergent-mediated 2D crystallization have been proposed [22], in line with the mechanisms evidenced at high lipid to protein ratios (Fig. 3). In mechanism I, the formation of the lipid layer and protein insertion occur simultaneously as binary and ternary micelles coalesce during detergent removal, followed by crystallization. In mechanism II, bilayer formation and protein incorporation are

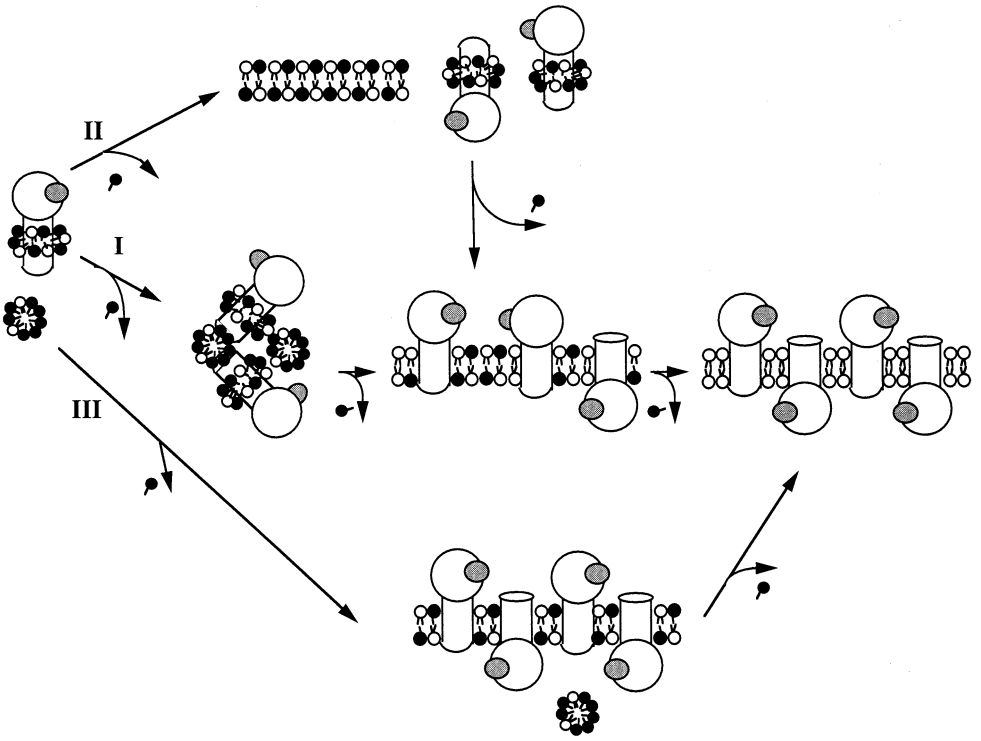

Fig. 3. Proposed mechanisms for 2D crystallization of membrane proteins in detergent-mediated reconstitutions. The scheme depicts possible sequences of events leading to 2D crystal formation upon detergent removal from a mixture of lipid–detergent and lipid–protein–detergent micelles. Mechanism I: the formation of the lipid layer and protein insertion occur simultaneously as the two kinds of micelles coalesce during detergent removal, followed by crystallization. Mechanism II: bilayer formation and protein incorporation are separate events. In this case, protein and lipid micelles dissociate separately forming first lipid bilayers. Then, the protein molecules insert into the pre-formed bilayers and finally crystallize. Mechanism III: crystal contacts are established when micelles are detergent-depleted, i.e. bilayer formation, protein insertion and 2D crystallization happen by a one-stage mechanism.

separate events. In this case, lipid–detergent and lipid–protein–detergent micelles dissociate separately forming first lipid bilayers. Then, the protein molecules insert into the pre-formed bilayers and finally crystallize. Finally, in mechanism III, crystal contacts are established when micelles are detergent-depleted, i.e. bilayer formation, protein insertion and 2D crystallization happen by a one-stage mechanism.

Mechanism III has been proposed for the 2D crystallizations of LC-II [60] and Ca-ATPase [42]. It was shown for these two proteins that, at the early beginning of detergent removal, lipid–detergent–protein micelles fused, leading to very small crystalline aggregates consisting of only few unit cells with the same lattice as large 2D crystals observed after total detergent removal. It can be stressed that these two membrane proteins have also been crystallized using the batch procedure, a 2D crystallization method in which micellar solutions are simply incubated at a given lipid–protein–detergent, without detergent removal [22]. Since the batch method is best interpreted as variations of 3D crystallization conditions, it could be suggested that, in 2D crystallization trials, mechanism III could apply to those membrane proteins with a high tendency to interact, aggregate or crystallize in micellar solutions.

Concerning mechanism II, it has been clearly demonstrated important in proteoliposome reconstitution. However, in such reconstitutions, due to the high lipid to protein ratios, the starting micellar solutions contain a huge excess of pure binary lipid–detergent micelles as compared to ternary protein–lipid–detergent micelles. Thus, a mechanism in which lipid–detergent micelles can dissociate separately upon detergent removal is highly probable. On the contrary, in 2D crystallization experiments, the lipid to protein ratios are very low, decreasing considerably the percentage of binary micelles and conse-

quently the probability of mechanism II in which bilayer formation can precede protein incorporation. However, such a mechanism may be taken into account in 2D crystallization trials where the lipids are pre-solubilized in a detergent before to be added to the solubilized protein. Due to the slow rate of exchange of lipids and proteins between micelles, such a mixing order imposes to start with two micelle populations, with a relatively small number of the protein–detergent micelles containing phospholipid. Such a starting solution can lead to mechanism II as suggested in a recent report on the 2D crystallogenesis of the glutathione transferase [61]. This points out for the importance of the way to mix lipid and protein in 2D crystallization trials: whether detergent-pre-solubilized lipids are added to the micellar protein solution or liposomes directly added to the micellar protein solution may lead to different mechanisms of 2D crystallization.

Concerning mechanism I, it seems to be the best model for most 2D crystallizations of membrane proteins. In this context, Dolder et al. [50] reported a comprehensive and careful analysis of the assembly of 2D crystals by quasi-elastic light scattering and electron microscopy (see also [25]). In these experiments, mixtures of lipid–protein–detergent were submitted to dilution while measuring the hydrodynamic radii of the aggregates formed by dynamic light scattering. Dilutions exhibited characteristic lambda-shaped dilution curves with intermediate rod-shaped particles that converted into large densely packed proteoliposomes. According to the 'three-stage' models of reconstitution and solubilization, the lambda-shaped curves corresponded to the micelle–bilayer transition delineated by the saturation and solubilization points. Interestingly, electron microscopy analysis of the structures formed during dilution revealed that formation of crystals occurred shortly after this phase transition. Thus 2D crystals form at the early stage of the micellar to lamellar transition by coalescence of detergent–protein micelles with lipid–detergent micelles and they are stabilized due to strong protein–lipid interactions. The reconstitution of the membrane protein in the bilayer by simultaneous micelle coalescence is without doubt the primary event, but whether crystallization of tightly packed proteins occurs during integration or at a later stage is often not clear.

As future prospects, it will be of special interest to distinguish parameters affecting the incorporation of the protein into the bilayer from those leading to crystallization. This would allow to control the three main steps of 2D crystallogenesis, namely bilayer formation, protein incorporation and crystallization. The structures formed during detergent removal should be systematically monitored to distinguish the incorporation of the protein from its crystallization. From the crystallogenesis studies reported above, it appears that 2D crystals of many membrane proteins can be observed at the early beginning of detergent removal, i.e. at the early beginning of the micelle to bilayer transition. This also implies that proteins have both to homogeneously incorporate in the bilayer but also to find homogeneous optimal interactions at this transition. These two simultaneous processes appear crucial to the outcome of 2D crystallization and may explain the difficulty for many proteins to efficiently crystallize. For those membrane proteins difficult to crystallize, one should consider bilayer formation separately from crystallization and improve the strategy of 'induced 2D crystallization' [22]. In this case, reconstitutions should be first performed at high lipid to protein ratios leading to proteoliposomes with proteins homogeneously incorporated and rather close packed. Then, in a second step, the crystallization could be induced by chemical agents such as vanadate [18,41], phospholipase A2 [62] or physical treatment such as lipid temperature transition [63].

4. 2D crystallization of membrane proteins on functionalized lipid layers

In view of the difficulties related to the 2D crystallization of membrane proteins through the classical reconstitution method by detergent removal, a considerable interest exists presently for designing innovative strategies. This would increase the chances of success and the number of membrane proteins amenable to structural analysis by electron crystallography.

The technique of 2D crystallization on lipid layer is based on a specific interaction between the protein and ligands coupled to lipid molecules incorporated in a planar lipid film at an air–water interface. This

method has proven to be a remarkably successful approach for 2D crystallization of different soluble proteins leading to important structural information. Protein crystallization has been described to proceed in three steps: (1) molecular recognition between soluble proteins and specific lipid ligands; (2) diffusion of lipid–protein complexes in the plane of the film; (3) self-organization of the complexes into 2D crystals (for reviews see [64–66]). The question as to whether this method was also applicable to membrane proteins was an open challenge. Indeed, in the absence of experimental data, a dogmatic drawback of this strategy was related to the presence of detergent injected with the purified protein and which was expected to disrupt the lipid layer. However, through a careful study, we have recently been able to produce on planar lipidic templates the first 2D crystals of two radically different membrane proteins, FhuA and F_0F_1 ATP synthase, demonstrating the feasibility of this new strategy for membrane proteins [67].

As stated before, crystallization on lipid layer first depends on binding of the protein to a lipid layer through specific protein–ligand interactions. Since many membrane proteins are presently overproduced and purified using recombinant proteins containing a stretch of continuous histidine residues (His-tag), a nickel-chelating lipid has been employed as a general adapter molecule that will link any His-tag protein [67–71].

Second, the lipid monolayer approach has been evaluated for 2D crystallization of membrane proteins, keeping in mind the major difficulty expected due to the detergent present with the solubilized membrane protein and which might interfere, up to solubilization, with the functionalized lipid layer. This drawback has been circumvented by: (i) Adapting the order of lipid and protein additions: the lipid layer has first to be formed on a buffer solution droplet, followed by protein injection in the subphase. (ii) Spreading an amount of lipids in slight excess to that needed for one single monolayer. This allowed a higher compression state of lipid at the interface, slowing down the penetration of the detergent into the lipid layer. Under these conditions, micelles of His-tagged membrane proteins could bind to the spread lipid layer and protected the lipid layer from detergent solubilization, up to several weeks. Furthermore, the data clearly indicated that binding of the protein–detergent micelles reduced drastically the fluidity of the lipid layer surface with an overall appearance of a frozen surface. One explanation for this unexpected observation could be that the high micelle concentration at the surface might induce specific viscous and even 'gel-like' phases, as observed in many lipid–detergent micellar solutions [56,58]. (iii) Removing the detergent from the adsorbed ternary micelles. Following addition of biobeads in the solution below the lipid layer, the structures bound to the spread lipid layer were continuously transformed into large continuous single bilayer domains of several hundreds of microns. After 1 day of incubation, some 2D crystalline patches were observed in the large reconstituted membrane which grew and coalesced to form large crystalline areas.

A tentative model for the mechanisms of 2D crystallization of membrane proteins on a lipid layer has been proposed, which should be a working model for other future successful crystallizations (Fig. 4). Such a model include three steps: (1) binding of protein–lipid–detergent micelles to the lipid; (2) reconstitution of a bilayer around the pre-bound proteins by detergent removal; (3) diffusion of the lipid–protein complexes and further 2D crystallization.

It has to be stressed that one critical parameter for successful crystal formation of membrane protein on lipid layer was the lipid content of the protein micelles injected in the sub-phase. It was proposed that the detergent removal step allowed the reconstitution of a lipid bilayer around the protein from these lipids and that recruitment of the lipids spread at the surface, if it occurred, was not enough for bilayer formation. Thus, as compared to the classical volume method of 2D crystallization by detergent removal, the lipid layer strategy involves also a step of reconstitution. This explains that the nature of the lipids and the lipid to protein ratio in the protein micellar solutions injected below the functionalized lipid layer will be crucial parameters for success and will have to be determined experimentally for each membrane protein.

In conclusion, this innovative strategy opens a new promising field for membrane protein structure determination since: (i) It may increase the chances of success to produce 2D crystals of proteins difficult to

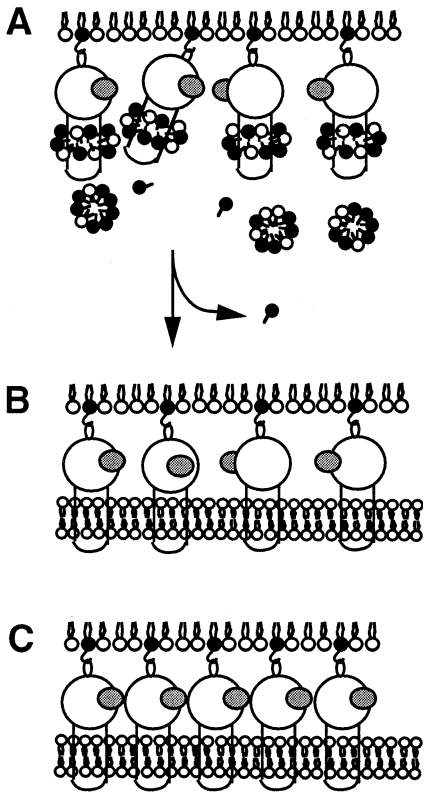

Fig. 4. Proposed mechanism for 2D crystallization of membrane proteins on a functionalized lipid layer (from [67]). (A) Binding of lipid/protein/detergent micelles to functionalized lipids at the air/water interface. (B) Reconstitution of membrane protein into a lipid bilayer upon detergent removal. (C) 2D crystallization.

crystallize by conventional volume method. In this context, it should be stressed that the specific adsorption of proteins to the lipid ligand in the first step of the model imposes a protein binding to the surface in a unique orientation and imposes 2D crystal formation with an unidirectional protein orientation. Such an orientation can be an advantage as opposed to the up and down orientation often observed in the 2D crystals produced by the classical detergent removal strategy. (ii) It allows to conduct structure analysis with smaller amounts of biological material. Indeed, since this strategy induces protein concentration at the interface instead of in volume this leads to the use of much less amount of proteins, below 1 μg protein as compared to 25–50 μg protein using the classical bulk method. This will be of particular help to laboratories studying receptors, channels and other membrane eukaryotic proteins which are currently at the early stages of developing procedures for overexpression and large-scale production. (iii) It would not require, in principle, high degree of protein purification since a highly specific protein binding to the lipid ligands may compensate this drawback.

As future prospects, an important effort should be devoted to the crystallogenesis process at the interface to understand and possibly improve the mechanism of formation and growth of 2D crystals through this strategy. In particular, it will be of interest to study the structures formed at the surface of the layer upon binding of protein micelles and the role of the different detergents in maintaining the stability of the lipid layer during the binding step. Finally, to make this strategy more general and/or more efficient, it will be also of importance to extend the layer method to a large range of detergents: presently, detergents with low cmc's such as Triton X-100, dodecylmaltoside or $C_{12}E_8$ have been used successfully and it will be important to analyze the behavior of high cmc detergents. Also important will be to extend the strategy to other specific molecular recognition between proteins and lipids at the surface, using other lipid-coupled physiological ligand or electrostatic interactions [64–66].

5. 2D crystallization of membrane proteins on a carbon support film

Surface assisted crystallization has been used in the past to generate 2D crystals of a variety of soluble proteins either at a liquid–liquid interface, on carbon-coated formvar film or carbon-coated grids [72–74].

In recent approaches, evaporation from a drop of solution for growing 3D crystals for X-ray analysis was found to produce large 2D crystals of the H^+-ATPase from Neurospora crassa, a 111 kDa integral membrane protein with a large 70 kDa hydrophilic domain [75]. In a first approach, 2D crystals were produced at the air–water interface of a drop of a dodecylmaltoside-solubilized membrane protein solution containing the precipitants PEG and ammonium sulfate. Such 2D crystals transferred to a carbon-coated grid for electron microscopic observation yielded a projection map at 10 Å resolution. However, the crystalline sheets were extremely fragile and the transfer resulted in complete disruption of the

crystal into small fragments. A second approach was then described by the same authors, by which 2D crystals of the protein were grown directly on the carbon film of a electron microscope support grid [76]. The experimental design resembled a conventional hanging drop vapor diffusion experiment except that a carbon-coated grid covered the drop surface.

Crystals grown by this strategy measured 5 μm across and had a thickness of 245 Å. The crystals formed after a few hours and longer incubation times resulted in the formation of multilayers. A three-dimensional structure was determined at 8 Å resolution [17] and revealed that the crystals consisted of tightly packed ATPase hexamers each surrounded by a toroidal micelle of the detergent. Indeed, the protein preparation did not contain lipid and no lipid was added to the crystallization mixture. Therefore, the 2D crystals described are fundamentally different from other 2D crystals of membrane proteins: there is no lipid bilayer in the surface crystals of the H^+-ATPase which are clearly not held together by hydrophobic interactions as in 2D crystals of most of the membrane proteins. Thus, the 2D crystals obtained by the surface crystallization method can be considered as 3D crystals consisting of detergent–protein mixed micelle complexes with a thickness of a single unit cell.

3D map revealed also that two protein layers were in contact via the small extracellular portion of the proteins, while the large 70 kDa hydrophilic cytoplasmic portion of this highly symmetric membrane protein faced either the carbon surface or the solution. Therefore, contacts to the carbon film are mediated by hydrophilic forces and the carbon film provides an interface at which molecules accumulate and rearrange, protecting the nuclei from disintegrating.

Another interesting observation was that temperature was critical in determining the packing of these 2D crystals (M. Auer, personal communication). When raising the temperature from 4°C to room temperature, tubular crystals of the H^+-ATPase were found at the air–water interface. It appeared in this case that these tubes were sheets that have rolled up into cylinders. They are characterized by the fact that the ends are broken off and are not nicely sealed as regular tubular crystals. Although this merits further confirmation it is tempting to relate the formation of dodecylmaltoside–protein tubular crystals to the tendency of this glycosylated detergent to form very long entangled micelles [58].

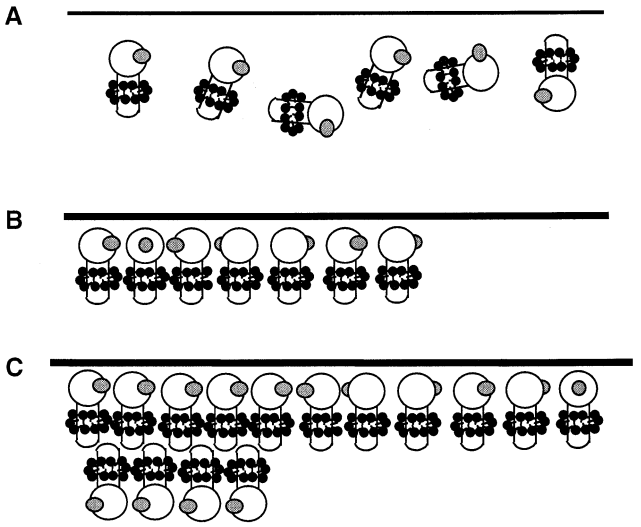

Fig. 5. Proposed mechanisms for surface crystallization on a carbon support film (from [76]). (A) The detergent-solubilized membrane protein diffuses freely in a drop solution for 3D crystallization. (B) Proteins adhere to the surface and diffuse along the surface into a close packed non-crystalline array. (C) The formation of a second layer of detergent–protein micelles locks the molecule of the first layer into a fixed orientation which results in the formation of a 2D crystal with a thickness of two protein molecules.

On the basis of all these observations, the authors proposed that the formation of double layer H^+-ATPase 2D crystals could occur in three stages (Fig. 5): (1) Initially the protein molecules diffuse freely in solution and adsorb to the carbon film. The rate of adsorption is higher than the rate of desorption as the hexamers clearly accumulate at the interface. (2) The protein hexamers then apparently diffuse along the carbon film and accumulate in a close packed non-crystalline array. (3) A second protein layer forms and large crystalline arrays were found, suggesting that the second layer assists in ordering of the first.

This new 2D crystallization strategy, which does not imply a detergent removal step, seems very promising in particular for those proteins for which conditions have been established for 3D crystallization. Indeed, the surface method seems to be rather specific for the 2D crystallization of those membrane proteins with a relatively large hydrophilic domain which tend to form 3D crystals more readily due to

the potential of extra-membrane portions for hydrophilic crystal contacts. Unlike classical 3D crystallization, the formation of 2D crystals is very quick and low material consuming. This would provide a way to grow 2D crystals of those membrane proteins that can only be obtained in small quantities and also specially if 3D crystals can be obtained but their quality or stability is insufficient for high resolution structure determination by X-ray crystallography. However, despite the fact that this strategy has been demonstrated to produce 2D crystals amenable to high resolution, more information is needed about the general use of this strategy related to the amphiphilicity of the protein and the nature of the detergent in inducing the surface crystallization of membrane protein micelles.

6. Conclusion

Despite major progress in the last few years, 3D crystallization of membrane proteins proved difficult and slow, and alternative approaches to structure determination became essential. In this context, electron crystallography, associated to 2D crystallization, is a technique that produced many structure determinations and is definitely a viable alternative strategy to X-ray crystallography. However, in order to make electron crystallography even more important as a tool for further structure determination of membrane proteins, enormous efforts are required: (1) For a deeper understanding of the 2D crystallogenesis process through analysis of the underlying molecular interactions and events during crystallization. This will rely on a comparative analysis using a large number of membrane proteins covering a wide spectrum of prototypical structures. From such studies we expect some general rules to be drawn concerning the role of the detergent, lipid, protein and to establish some physico-chemical rules in 2D crystallogenesis that can be applied to a broad range of membrane proteins. Some of the mechanisms elucidated in the case of lipid–detergent and lipid–protein–detergent systems may help in understanding the mechanisms of 2D crystallization. In addition, the techniques and methodologies adapted for the study of the molecular and supramolecular mechanisms of micelle to vesicle transition may be as well used for analyzing the process of 2D crystallization. (2) For new approaches to be evaluated, refined and applied to available proteins in order to increase the chances of success. This will enhance the success rate of 2D crystallization experiments and increase the number of membrane proteins amenable to high resolution structural analysis by electron crystallography.

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