Minireview

Two-dimensional crystallization of membrane proteins: the lipid layer strategy

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Received 11 July 2001; accepted 23 July 2001

First published online 7 August 2001

Edited by Andreas Engel and Giorgio Semenza

Abstract Due to the difficulty to crystallize membrane proteins, there is a considerable interest to intensify research topics aimed at developing new methods of crystallization. In this context, the lipid layer crystallization at the air/water interface, used so far for soluble proteins, has been recently adapted successfully to produce two-dimensional (2D) crystals of membrane proteins, amenable to structural analysis by electron crystallography. Besides to represent a new alternative strategy, this approach gains the advantage to decrease significantly the amount of material needed in incubation trials, thus opening the field of crystallization to those membrane proteins difficult to surexpress and/or purify. The systematic studies that have been performed on different classes of membrane proteins are reviewed and the physico-chemical processes that lead to the production of 2D crystals are addressed. The different drawbacks, advantages and perspectives of this new strategy for providing structural information on membrane proteins are discussed. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Two-dimensional crystallization; Membrane protein; Lipid layer; Lipid ligand

1. Introduction

Genomic studies of prokaryotic and eukaryotic organisms demonstrated that membrane proteins represent about 25% of the predicted proteins [1]. These results emphasize the importance of membrane proteins in many biological processes essential for life. However, compared to other protein classes, their functions are poorly understood due to the lack of structural information which precludes structure–function relationships to be established at a molecular level. For instance, less than 30 original structures have been solved to atomic resolution, a number which lags far behind that of soluble proteins, with several thousands accurately determined structures.

Three main bottlenecks may explain the difficulties in getting structural information on membrane proteins. First, membrane proteins are difficult to overexpress and only few membrane proteins are available in a large amount enough for structural analysis. Expression levels in mammalian, insect cells, oocytes, yeast or *Escherichia coli* still need to be improved for routine crystallization trials [2]. Production of protein in non-functional form as inclusion bodies and subsequent refolding may be a possible route towards realizing this goal [3,4]. Second, solubilization and purification of membrane proteins necessitate the use of detergents and require a difficult and empirical biochemistry. Although the addition of polyhistidine motifs by gene modification that specifically bind nickel-chelated nitriloacetic acid (Ni^{2+}-NTA) resins has greatly improved the purification step, it remains that the purity, the final concentration, the monodispersity, and the stability of the purified protein preparations are still limiting factors in further crystallization trials. Third, amphiphilic membrane proteins are notoriously difficult to crystallize and therefore the production of three-dimensional (3D) or two-dimensional (2D) crystals remains one of the major challenges to get structural information.

While conventional 3D crystallization has provided the majority of atomic structures of membrane proteins, success in growing 3D crystals in detergent micelles remains relatively infrequent [5]. This drawback is mainly related to the difficulty in maintaining a crystal lattice through the sole interactions between the hydrophilic domains of the proteins, the hydrophobic domains being shielded by the detergent micelles. To help with this problem, extensive works have been performed to develop new strategies. In particular, a novel approach has been introduced by Michel's group in which monoclonal antibody fragments were specifically bound to proteins, increasing the interactions between the hydrophilic domains of membrane proteins [6]. More recently, 3D curved membranes have been devised as crystallization matrices with the idea that proteins in such membrane mimetic systems should be more stable than in micelles. Highly viscous, bi-continuous lipidic cubic phases have been successfully used as matrices for nucleation and further 3D crystallization of different proteins [7,8].

As a viable alternative to the 3D crystallization of membrane proteins in detergent micelles, the reconstitution of membrane protein into lipidic membranes to form crystals confined to two dimensions has opened a new way to solve structures by electron crystallography. If only three membrane protein structures have been resolved to atomic resolution [9–11], more than 20 membrane proteins have been resolved to medium resolution (4–8 Å) in 3D maps or projections in

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Abbreviations: Ni^{2+}-NTA, nickel-chelated nitriloacetic acid; 2D, two-dimensional; 3D, three-dimensional; cmc, critical micellar concentration; His, histidine; RLM, reflected light microscopy

PII: S0014-5793(01)02748-X

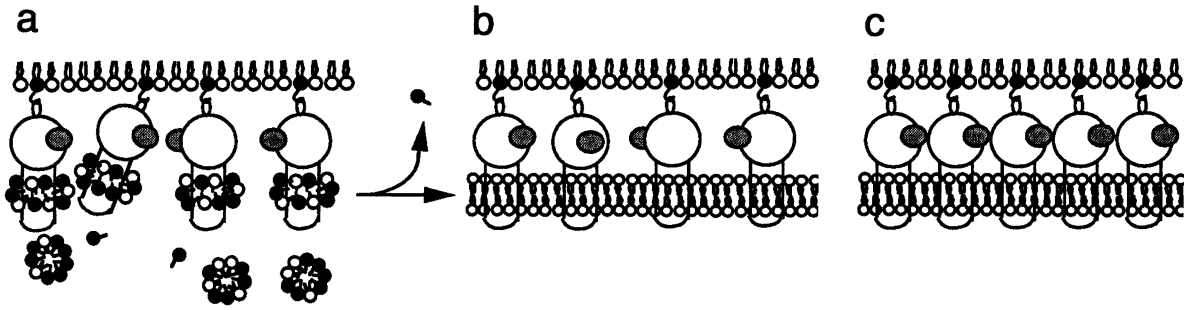

Fig. 1. Mechanism for 2D crystallization of membrane proteins on lipid layer at the air/water interface. (a) Binding of protein/lipid/detergent micelles to the functionalized lipid layer spread at the air/water interface. (b) Reconstitution of proteins into a lipid bilayer upon detergent removal. (c) 2D crystallization.

plane. Although of medium resolution, these 2D crystals have revealed the secondary structures of different membrane proteins not yet crystallized in 3D. Furthermore, they allowed to analyze structural similarities within protein families [12] or large conformational changes induced by different substrates [13]. However, as for 3D crystallization, the production of well ordered 2D crystals remains rare and many proteins continue to resist efforts.

Except for few examples of 2D crystals of membrane proteins in native membranes, 2D crystals are usually obtained by the general method of detergent-mediated reconstitution of membrane protein [14]. The principle involves detergent removal from micellar detergent solutions containing the purified protein and a suitable combination of lipids. In addition to the large number of parameters that critically affect the production of 2D crystals (e.g. temperature, buffer composition, lipid to protein ratios, nature of the ternary micelle components), detergent removal is a key step since it controls the micelle to bilayer phase transition, the protein incorporation into the bilayer and its crystallization. A number of excellent reviews are available concerning the different methods to produce 2D crystals and the physico-chemical mechanisms leading to their formations [15–20].

In the present paper we will focus on a new experimental approach for 2D crystallization of membrane proteins that have allowed promising perspectives to be foreseen. By combining the concepts of surface recognition and 2D crystallization of membrane proteins by detergent removal, it has been possible to crystallize membrane proteins previously bound to a lipid layer spread at the air/water interface. As a main result, such a new strategy has been demonstrated efficient for crystallization of radically different membrane proteins [21]. Besides to represent an alternative strategy for 2D crystallization, this new approach gains the advantage to decrease significantly the amount of material needed in incubation trials, opening the field of crystallization to those membrane proteins difficult to surexpress and/or purify.

2. Principle Of 2D crystallization on lipid layer

The principle of the lipid layer 2D crystallization method, initially developed by the group of Kornberg, is based on the specific interaction between a soluble protein and a lipid ligand inserted in a planar lipid film at an air/water interface [22–25]. This method has been applied to more than 20 soluble proteins leading, for some of them, to important structural information to 3 Å resolution [26,27]. 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 proteins into 2D crystals. It is important to stress that one important advantage of this method, related to the binding step and the concentration of the protein at the surface, is that only a small amount of protein is required, less than 1 μg per incubation trial.

The question as to whether this strategy was applicable to membrane proteins was an open challenge. Indeed, its use for membrane proteins was confronted to the indispensable presence of detergent with the protein, which was expected to alter the integrity of the lipid film. However, through careful studies, the feasibility of this approach to crystallize membrane proteins was recently demonstrated. 2D crystals of radically different membrane proteins have been produced including TF0F1, the ATP synthase from thermophilic bacteria PS3, FhuA, a highly specific porin from *E. coli* [21], and, more recently, bacteriorhodopsin and the ADP/ATP transporter Anc2 from yeast mitochondria (Chami et al., in preparation) as well as aquaporin Aqp1 (S. Scheuring, personal communication) and a plant H^+ ATPase [28].

Comparative and systematic studies allowed to identify the basic principles controlling the formation of membrane protein 2D crystals on lipid layers and a working model has been proposed. Such a model includes three steps (Fig. 1): (1) binding of protein micelles to a surface lipid layer through specific or electrostatic interactions; (2) reconstitution by detergent removal of a lipid bilayer around the previously bound protein micelles; (3) further 2D crystallization in the reconstituted lipid bilayer.

3. The three steps for membrane protein crystallization on lipid layer

3.1. Binding of the protein micelles to the lipidic surface

One of the main difficulties expected in the development of the lipid layer to crystallize membrane proteins was the presence of the detergent during the binding step. During this step, the detergent has to be at a concentration above its critical micellar concentration (cmc) to maintain the protein amphiphilicity and may interfere, up to complete solubilization, with the lipid layer spread at the surface. Indeed, spreading a lipid layer directly on a detergent-containing droplet proved to be unsuccessful due to an immediate solubilization of the surface lipid layer.

This drawback has been circumvented: (i) by adapting the

order of lipid and protein additions: the surface lipid layer has first to be formed on a drop of a detergent-free buffer solution, followed by injection in the sub-phase of the protein in a micellar form; (ii) by spreading an amount of lipids in slight excess (10–20%) to that needed for a single monolayer. This allows a maximal compression state of lipid at the interface, slowing down the penetration of the detergent into the pre-formed lipid monolayer. Under these conditions, when a detergent was injected at a concentration well above its cmc, a gradual solubilization of the spread lipid layer was shown to occur, but in more than 4 h, a process significantly longer than the solubilization process of lipids in bulk [14,29,30]. Interestingly, when the same amount of detergent was injected but with the protein, the lipid layer was extremely stable and no solubilization was observed, even after 1 month incubation at 4°C. Although a comprehensive explanation of this unexpected stability remains speculative, it is likely related to the binding of the protein which leads to a high concentration of protein micelles at the interface. This high micelle concentration could induce, as already observed in many lipid–detergent micellar solutions [31–33], specific viscous and even 'gel-like' phases which in turn would slow down detergent diffusion in the lipid layer. Such an interpretation is in agreement with the observed appearance of a frozen surface upon binding of the protein–detergent micelles to the lipid layer surface (see below).

Under the experimental conditions defined above for the binding step, it has been possible to bind several membrane protein micelles to different functionalized lipid layers. The structures formed on the interfacial film were sufficiently stable to be transferred to carbon grids and analyzed by transmission electron microscopy. They appeared as thin planar domains which can be several tens of μm in size (Figs. 2a,b and 3a). At high magnification, a protein-like grain was clearly visible in these domains, denoting the efficient binding of the proteins. When the proteins were large enough to be visualized, the bound micelles appeared densely packed (Fig. 3b) and, in few cases, optical diffractions showed hexagonal patterns typical of a highly close packing (Fig. 2c). However, longer incubations, up to several weeks, never led to the formation of crystalline lattices of these protein micelles bound to the lipid layer.

A point to be stressed is that the efficiency of the binding step was found to be specific for detergents with low cmc values such as dodecylmaltoside, Triton X-100 or other polyoxyethyleneglycol detergents. Use of high cmc detergents, including octylglucoside, or bile salt derivatives, always failed due to a rapid solubilization of the lipid layer. Whatever, for those membrane proteins purified in high cmc detergents, the difficulty could be overcome: (1) by exchanging the high cmc detergent for a low cmc detergent using suitable chromatographic columns; (2) by simply diluting the protein in a low cmc detergent during the injection step; or (3) by using a new type of lipid layer made of fluorinated chains. Such fluorinated lipids, which increase drastically the hydrophobicity of the lipid layer, should prevent its solubilization whatever the nature of the detergent injected in the sub-phase [28].

3.2. Reconstitution of pre-bound proteins into a lipid bilayer

As stated above, 2D crystallization of protein micelles bound to the lipid layer has never been observed. This is probably related to the difficulty to create hydrophilic contacts between membrane proteins embedded into micelles as reported for 3D crystallization trials. Thus, to favor protein–protein contacts, detergent has to be removed, with the idea to reconstitute a lipid bilayer around the previously bound protein, in order to maintain its amphiphilicity. Detergent removal was performed, in about 4 h, through successive additions of Bio-Beads SM2 into the solution below the lipid layer [34,35].

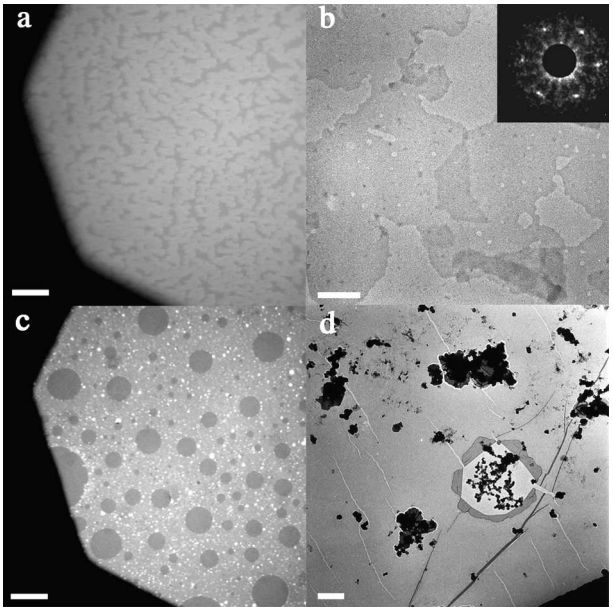

Fig. 2. 2D crystallization of His-FhuA on Ni-NTA lipid layer. Experimental conditions as in [21]. RLM experiments have been performed with a Leica DMR microscope equipped with a long distance objective (×20, 0.40 nominal aperture). Images of the the surface of the wells were recorded without any labelling by a non-intensified CCD camera (COHU 5100) without further electronic improvement to increase the contrast. Upper part: binding step. (a) Surface of the incubation well observed by RLM showing the protein micellar domains (light gray) formed after protein binding. Bar: 50 μm. (b) High magnification electron micrograph of the transferred micellar domains. Bar: 200 nm. Inset: computed diffraction pattern showing the close packing of micellar proteins. Lower part: reconstitution step. (c) Surface of the incubation well observed by RLM after detergent removal from a. Reconstituted membranes correspond to the light gray background with defects appearing as dark gray circles. Bar: 50 μm. (d) Low magnification electron micrograph of the membrane sheets formed at the interface after detergent removal. The large membrane is a single bilayer as shown by the folded sheet. Bar: 2 μm.

It has been shown that the reconstitution of a lipid bilayer around the pre-bound protein was strictly related to the presence of lipids in the initial micellar protein solutions injected in the sub-phase. When lipids are added to the solubilized protein before injection, large bilayered membranes could be reconstituted while, in the absence of added lipids, only aggregated structures were found at the surface after detergent removal. Thus detergent removal allows membranes to be reconstituted from the lipids present in the ternary lipid/protein/detergent micelles bound to the functionalized surface. At this point, it has to be noted that although reconstitution of the bilayer is independent of the nature of the lipid added to the initial micelles, the lipid specificity is crucial for the following 2D crystallization step.

Electron microscopy analysis of the transferred protein–lipid films indicated that the domains formed after the binding of

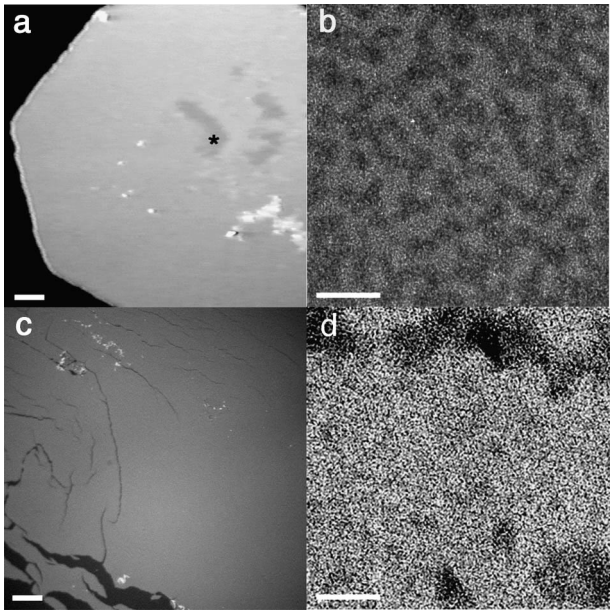

Fig. 3. 2D crystallization of His-TF0F1 on Ni-NTA lipid layer. Experimental conditions as in [21]. Upper part: binding step. (a) Surface of the incubation well observed by RLM showing the protein micellar domains formed after protein binding (light gray) and unbound areas (*). Bar: 50 μm. (b) High magnification electron micrograph of the transferred micellar domains. Bar: 200 nm. Lower part: reconstitution step. (c) Surface of the incubation well observed by RLM after detergent removal from a. Reconstituted membranes correspond to the light gray background with defects appearing as dark gray breaks. Bar: 50 μm. (d) High magnification electron micrographs of the membrane sheets formed at the interface and showing crystalline areas of the reconstituted proteins. Bar: 100 nm.

protein micelles were continuously transformed into very large membrane sheets during detergent removal. The large reconstituted membranes were several tens of μm in size, covering nearly all the electron microscope grid (Figs. 2c and 3c). Edge views, observed when lipid layer folded away from the grid, demonstrated that this continuous layer consisted of a single bilayer (Fig. 2d). In addition, observations at high magnification clearly indicated the proteins in a densely packed or near crystalline arrangement in the membranes, denoting the efficiency of the reconstitution process (Fig. 3c). Finally, the morphology of the membranes reconstituted at the surface was always large planar sheets but never vesicles or tubes as often observed in 2D crystallization trials in volume.

3.3. 2D crystallization

After binding of the ternary lipid–detergent–protein micelles and complete detergent removal, proteins make contacts in the reconstituted membrane and, in favorable cases, can crystallize as observed for FhuA, TF0F1, bacteriorhodopsin and Anc2.

Besides the nature of the protein, critical parameters for successful crystal formation were, as for the 2D crystallization in volume, the lipid to protein ratio and the nature of the lipid added to the micellar protein preparation before injection in the crystallization well. Noteworthy, lipid to protein ratios higher than those used in the volume method were needed to obtain large reconstituted membranes at the surface. Indeed, while reconstitution in volume involved both lipid–detergent and lipid–protein–detergent micelles, only the latter were bound to the functionalized lipidic surface and participated in the reconstitution step. Other parameters that have to be adjusted experimentally for successful crystallization are related to the temperature, viscosity and buffer composition.

Concerning the quality of the 2D crystals obtained so far, it has to be stressed that the lipid layer method generally produced very large crystalline areas, much larger than those generally produced by the conventional bulk method. However, highly coherent areas in the reconstituted crystalline domains were too small to extract high resolution structural information and resolutions of the best crystal extend to about 10 Å. One interpretation of this drawback could be related to the major problem encountered with the the lipid layer method, that is the difficulty in the transfer of interfacial films which can alter the crystallinity of the structures formed in situ. However, since several large coherent 2D crystals have been obtained with soluble proteins, the small size of highly coherent crystals of membrane proteins cannot be related to unfavorable properties of the interface and its transfer. It is thus clear that for further improvements of the method, crystallogenesis studies are needed to understand the restricted growth of coherent membrane protein 2D crystals formed at the surface. In particular, a limiting factor could be a limited freedom for proteins to establish crystalline contacts in a reconstituted membrane once bound to a lipid ligand at the surface.

Concerning the organization of the proteins in the 2D crystals, all 2D crystals obtained with the lipid layer method have shown proteins incorporated in the same orientation. Such specificity is the result of the unidirectional orientation imposed by the specific binding of the protein to its lipid ligand. This has been clearly demonstrated in the case of FhuA, a protein for which 2D crystals have been produced through the use of the two strategies: while the classical 2D crystallization in volume led to 2D crystals with proteins facing up and down in the plane of the membrane [36], the lipid layer led to 2D crystals with all FhuA molecules in the same orientation (Fig. 4). This result also pointed out that 2D crystals obtained at the surface could not be related to 2D crystals formed in the volume of the droplet and then absorbed and fused to the lipid layer. Finally, the asymmetric protein orientation imposed during the binding step has been demonstrated to be an advantage in 2D crystallization trials of TF0F1, a protein which organizes in up and down orientations using crystallization trials in volume. Although of low resolution, 2D crystals of TF0F1 produced with the lipid layer strategy represent the best 2D crystals obtained so far for the whole enzyme [21]. It is likely that hydrophilic contacts through adjacent F1 parts, which are the sole protein/protein interactions that may occur in an asymmetric orientation, are more favorable for crystallization than hydrophilic and additional hydrophobic interactions that may occur in the up and down orientations.

4. Lipid ligands

Interfacial lipid films used for growing protein 2D crystals contain a specific lipid ligand and a second lipid, referred to as dilution lipid made of an unsaturated phospholipid to ensure a lipid layer in a fluid state. Mixtures of ligand lipid/dilution lipid ranging from 0.75 to 0.25 (mol/mol) are frequently used.

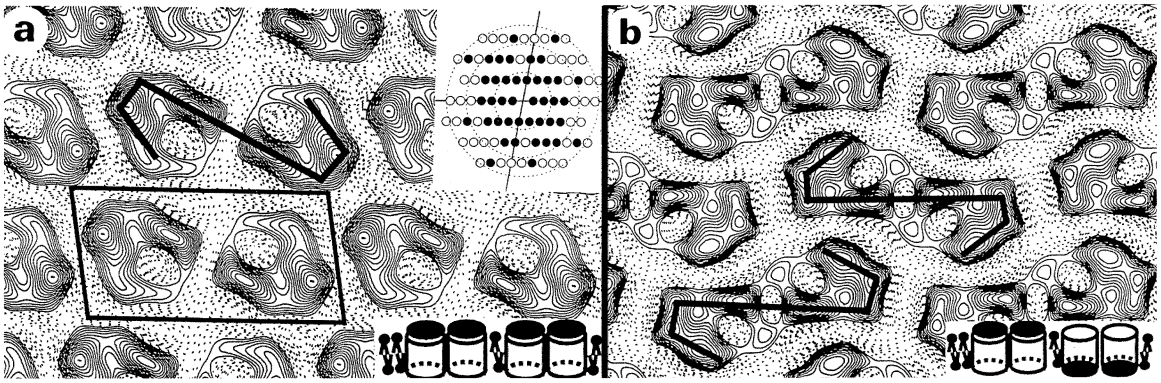

Fig. 4. Protein orientation in different 2D crystals of FhuA. (a) Average projection map from 2D crystals produced by the lipid layer method [21]. Inset: reflections correspond to IQ 1 (●) and IQ 2–4 (○) and rings to 1/40, 1/20, 1/15 $Å^{-1}$. (b) Average projection map from 2D crystals produced by the method of 2D crystallization in volume [36]. Projection maps calculated at 15 Å resolution. FhuA dimers are delineated in each map by an S line. The schemes indicate the orientations of FhuA dimers in both 2D crystals.

The protein to be crystallized may interact with the lipid via non-specific electrostatic interactions or via specific interactions mediated by high-affinity ligands attached to the polar head group of the lipids. To date, most of the experiments reported for 2D crystallization of membrane proteins have used a lipid derivatized with a Ni^{2+}-NTA head group molecule to link genetically modified recombinant proteins containing an accessible stretch of histidine (His) residues. This lipid ligand already proven successful for 2D crystallization of several His-tagged soluble proteins [37–41] has also been demonstrated efficient for membrane proteins [21,28]. Buffer conditions for the binding of His-tagged proteins to Ni^{2+}-NTA lipids have been reported similar to those reported for binding of proteins to Ni^{2+}-NTA resins. In particular, the specific binding of His-tagged protein to the Ni^{2+}-chelating lipid required low imidazole concentrations, the absence of EDTA and pH above 6.0. Differences in the kinetics of the binding of proteins to Ni-NTA lipids were observed, likely due to different accessibility of the His-tag. For example, binding occurred in less than 1 h at 20°C for proteins with a His-tag attached to a large external loop, as for $(His)_{10}$ TF0F1 or $(His)_6$ FhuA, while overnight incubation at 4°C was needed for binding more hydrophobic proteins with a His-tag attached to a short C-terminus, as for $(His)_6$ melibiose permease or $(His)_6$ Anc2. For protein concentrations below 100 nM (10 μg/ml for a 100 kDa protein) no binding was observed, in agreement with the values of dissociation constants between 70 and 200 nM measured on immobilized Ni^{2+}-NTA surface [42].

More recently, binding and 2D crystallization of membrane proteins have been extended to non-specific electrostatic interaction using charged lipids as lipid ligands. For example, solubilized bacteriorhodopsin has been crystallized after binding to positively charged lipid layer leading to 2D crystals diffracting to 10 Å resolution in ice (Fig. 5a). On the other hand, 2D crystals of the mitochondrial transporter, Anc2, have been produced after binding to negatively charged surface (Fig. 5b), with even better reconstitution than that obtained with a Ni-chelating surface, likely due to the low accessibility of the His-tag of this protein. Thus, electrostatic binding of a protein to positively or negatively charged lipids can be adapted depending upon the charges distribution of the membrane proteins and opens the field of lipid layer crystallization to those membrane proteins that cannot be genetically modified.

5. Experimental set-up and reflected light microscopy (RLM)

Experiments are done in 60 μl teflon wells, with a 4 mm diameter, i.e. the same size as the electron microscopy carbon-coated grids which are deposited on the crystallization drop for the transfer of the structures formed at the interface. On the side of each well, an injection hole allows addition of micellar protein solutions or Bio-Beads under the lipid layer.

To follow the binding to the surface and the 2D crystallization steps, several techniques have been used in the case of soluble proteins, including epifluorescence, ellipsometry, surface pressure measurement, viscosimetry, Brewster angle microscopy and electron microscopy [43–46]. However, these approaches require large wells and are material consuming, precluding their use in 2D crystallization trials of membrane proteins. We have found that RLM was a powerful tool to follow the important steps of protein binding and reconstitution at the interface. Furthermore, observations are performed directly on the surface of the crystallization wells and allow a fast screening in situ of the crystallization parameters (Chami et al., in preparation).

After spreading the lipid layer on the detergent-free buffer solution, the surface focused by RLM appeared as a homogeneous gray background except for some small aggregates of lipid floating at the surface. The easily observed movement of these lipid aggregates is in agreement with the fluid state of the spread lipid layer. Following injection of a micellar protein solution, significant changes of the surface can be observed: (i) the surface fluidity decreased and appeared frozen with no more movement; (ii) the surface is progressively covered by whiter areas (Figs. 2a and 3a) which increased in size, covering nearly all the surface of the well. Control experiments with His-tagged proteins demonstrated that these optical changes were correlated to the specific binding to Ni^{2+}-NTA lipids since they were not observed in the presence of high imidazole concentrations or in the presence of EDTA. In addition, after transfer of the surface to carbon-coated grids, the white areas observed in RLM corresponded clearly to the protein-containing planar domains observed by electron microscopy (Figs. 2b and 3b).

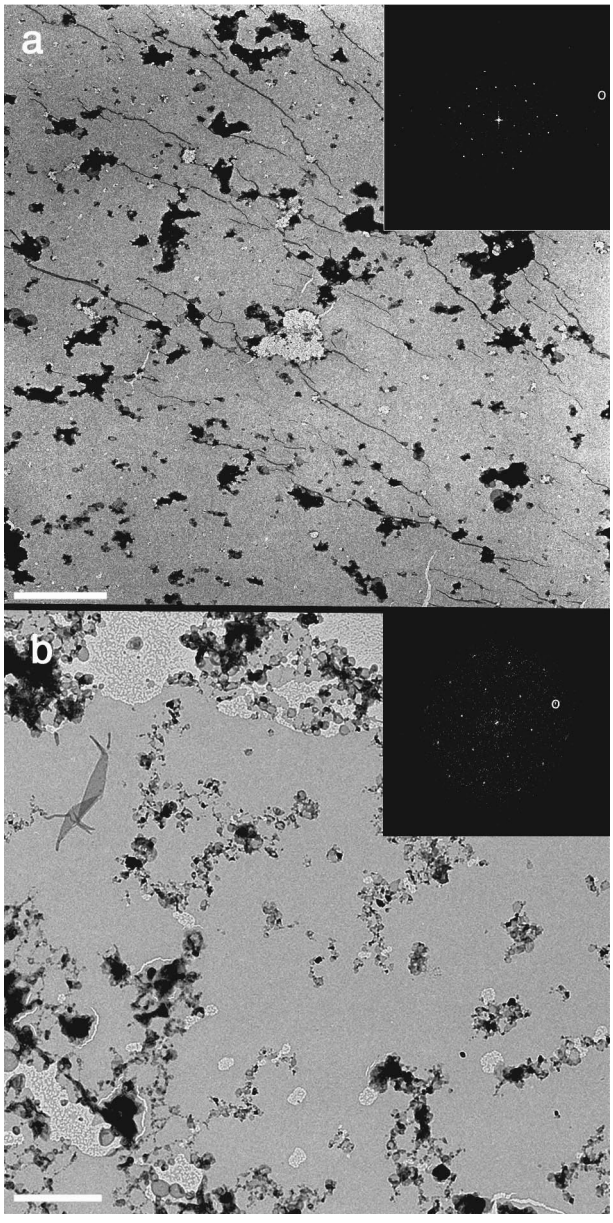

Fig. 5. 2D crystallization on charged lipidic surface. (a) Electron micrograph of the 2D crystals of bacteriorhodopsin produced on positively charged lipid layer. A mixture of delipidated bacteriorhodopsin solubilized in dodecylmaltoside was supplemented with egg phosphatidylcholine, injected and reconstituted at 25 μg/ml beneath a lipid layer made of 1,2-dioleoyl-3-dimethyl-ammonium propane. Bar: 5 μm. Inset: optical diffraction pattern with spots up to 10 Å in ice (○). (b) Electron micrograph of the 2D crystals of Anc2 produced on negatively charged lipid layer. A mixture of His Anc2 solubilized in dodecylmaltoside was supplemented with egg phosphatidylcholine, injected and reconstituted at 30 μg/ml beneath a lipid layer made of egg phosphatidic acid. Bar: 2 μm. Inset: optical diffraction pattern with spots up to 18 Å in ice (○).

Upon detergent removal, the contrast of the surface focused by RLM increased drastically, concomitant with the transformation of the micellar layer into reconstituted bilayer. Reconstituted membrane appeared by RLM as large light gray areas covering all the crystallization well, i.e. 4 mm diameter (Figs. 2c and 3c). Interestingly, again there was a good correlation between the morphology and the size of the structures observed by RLM and the structures observed by electron microscopy after transfer of the lipid layer. Thus, despite RLM provides information on a microscopic range, it represents a powerful tool to visualize in situ two important steps of the crystallization process, i.e. binding and reconstitution by detergent removal. The kinetics of both processes can be controlled through the structures observed by optical microscopy and some parameters of the reconstitution adjusted without the need for time consuming electron microscopy observations.

6. Conclusions and perspectives

Membrane proteins have been poorly characterized compared to soluble proteins, because they are embedded in a lipidic environment and thus require special treatment to be studied. This hindrance is clearly illustrated by the few number of high resolution structural data on membrane proteins, itself related to the difficulty in crystallizing these membrane proteins. Thus, a considerable interest exists for designing innovative strategies to produce 2D or 3D crystals of membrane proteins that are amenable to structural analysis by electron or X-ray crystallography.

This manuscript reports on the lipid layer 2D crystallization strategy which can be foreseen as a new promising alternative to the conventional method for membrane protein 2D crystallization. This strategy shown recently efficient for the crystallization of a very diverse range of membrane proteins can now be run in parallel to the classical method in volume and will increase the chances of success. Further detailed studies of crystallogenesis are currently in progress to improve the strategy in terms of producing highly coherent 2D crystals and to demonstrate its general applicability, using other membrane proteins solubilized in different classes of detergent.

Although, as other crystallization methods, the lipid layer method has its own disadvantages related to reproducibility, quality of the transfer and size of highly coherent 2D crystals, it gains the important advantage to require very small amounts of proteins per crystallization trials. Thus this method opens the field of 2D crystallization to membrane proteins which are produced and purified in low amounts and will be of particular help for studying receptors, channels and other membrane eukaryotic proteins which are, to date, difficult to overexpress. In addition, although the vast applicability of the NTA lipid already provides a common moiety for the binding of many diverse His-tagged membrane proteins, the possibility to extend the lipid layer method to other specific affinity binding is expected to make this strategy even more general. For example, the use of non-specific electrostatic interactions between membrane proteins and charged lipids has already been demonstrated efficient. Interestingly, several functionalized lipids already developed to bind soluble proteins could also be used such as lipids with a head group made of ATP analogues to bind proteins with accessible nucleotide binding sites [47], biotin group for avidin genetically modified proteins [48] or *N*-ethylmaleimide groups for cysteine-containing proteins [49].

Finally, further development of the lipid layer strategy for providing structural information on membrane proteins would be: (1) to take advantage of the flatness of the lipid surface which provides the formation of large and planar reconstituted membranes. Such reconstituted membranes in which proteins are incorporated in a preferred orientation

and at high density would be a suitable material for high resolution atomic force microscopy [50]; (2) to use the lipid layer as a template for fusion of pre-formed small 2D crystals [51]. Preliminary experiments have shown the feasibility of this approach by fusing, on a positively charged lipid layer, small vesicular 2D crystals of the mechanosensitive channel MscI produced by detergent removal in volume; (3) the use of functionalized lipid layers to concentrate protein micelles at an interface and that can serve as seeds for epithaxial growth in membrane protein 3D crystallization trials, as it has been demonstrated in the case of soluble proteins [52].

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