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Binding, reconstitution and 2D crystallization of membrane or soluble proteins onto functionalised lipid layer observed in situ by reflected light microscopy

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ABSTRACT

Monolayer of functionalized lipid spread at the air/water interface is used for the structural analysis of soluble and membrane proteins by electron crystallography and single particle analysis. This powerful approach lacks of a method for the screening of the binding of proteins to the surface of the lipid layer. Here, we described an optical method based on the use of reflected light microscopy to image, without the use of any labeling, the lipid layer at the surface of buffers in the Teflon wells used for 2D crystallization. Images revealed that the lipid layer was made of a monolayer coexisting with liposomes or aggregates of lipids floating at the surface. Protein binding led to an increase of the contrast and the decrease of the fluidity of the lipid surface, as demonstrated with the binding of soluble Shiga toxin B subunit, of purple membrane and of solubilized His-BmrA, a bacterial ABC transporter. Moreover the reconstitution of membrane proteins bound to the lipidic surface upon detergent removal can be followed through the appearance of large recognizable domains at the surface. Proteins binding and reconstitution were further confirmed by electron microcopy. Overall, this method provides a quick evaluation of the monolayer trials, a significant reduction in screening by transmission electron microscopy and new insights in the proteins binding and 2D crystallogenesis at the lipid surface.

1. Introduction

Two-dimensional crystallization on functionalized lipid layer combined with the electron crystallography is a useful and interesting approach in structural biology. The principle relies on the spreading at the air/water interface of a lipid layer that could interact with proteins present in the volume. The lipid and protein interactions can be mediated though electrostatic interactions between lipid and protein of opposite charges. These interactions can also be highly specific such as between proteins and their native lipidic receptor, between proteins and any lipid ligand specially designed, or between His-proteins and a lipid ligand bearing a Ni^{++}-NTA head group (for a recent reviews see (Dietrich and Venien-Bryan, 2005; Kelly et al., 2010)). Following the binding step, protein/protein interactions in the surface plane can lead to the formation of 2D arrays of proteins.

This approach has provided structural information from 2D crystals of toxins bound to their lipid receptors (Ellis et al., 1997; Hagnerelle et al., 2002; Mosser et al., 1992), of 2D crystals of proteins resistant to 3D crystallization, e.g. polymerases and viral proteins (Barklis et al., 1998; Darst et al., 1991), and of 2D paracrystals of cytoskeleton proteins (Taylor and Taylor, 1999). This approach has also been extended to the 2D crystallization of membrane proteins. In this case, after the binding of membrane proteins solubilized in micelles of detergent to the functionalized surface, detergent was removed and proteins were reconstituted into a new lipid bilayer suspended at the surface (Arechaga and Fotiadis, 2007; Lebeau et al., 2001; Lee et al., 2008; Levy et al., 2001; Levy et al., 1999). Recently, functionalized lipid surfaces have been used for the 3D reconstruction by single particles analysis of bound proteins (Kelly et al., 2008; Kelly et al., 2010).

The functionalized lipid layer at the air/water interface provides remarkable specificities compared to the experiments performed in volume: (i) the surface concentrates proteins present in the volume decreasing the amount of proteins required per assay up

Abbreviations: DDM, *n*-dodecyl-β-D-maltoside; DOPC, dioleoylphosphatidylcholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; Ni^{++}-NTA DOGS, 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid)succinyl] (nickel salt); Gb3, globotriaosylceramide; EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; EM, electron microscopy; RLM, reflected light microscopy; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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to nanograms. (ii) The specific recognition between proteins and lipid ligand allows to purify proteins at a level suitable for single particle analysis (Kelly et al., 2008) or to obtain 2D crystal of cholera toxin B-subunit in presence of an excess of contaminant (Mosser and Brisson, 1991a). (iii) In the case of 2D crystallization of membrane proteins, the binding step imposes a unique orientation of proteins within the reconstituted bilayer as found in native membranes. The unidirectional orientation also favors the 2D crystallization of proteins with large extramembraneous domains like for F0F1 that otherwise often led to stacked 2D crystals after reconstitution in volume (Arechaga and Fotiadis, 2007; Levy et al., 1999). (iiii) Finally, the presence of a lipid layer may protect fragile macrocomplexes sensitive to the surface tension of the air/water interface (Kelly et al., 2008).

The process of binding and of 2D crystallization of proteins has been analysed by several approaches directly in the 2D crystallization wells or in the Langmuir trough. Analysis in crystallization wells was usually performed by electron microscopy (EM). After transfer of the surfaces onto microscopy grids, the increasing density of proteins and the nucleation of 2D crystals were observed (Mosser and Brisson, 1991a). However since surfaces have to be transferred for the observation by EM, the kinetics of protein binding can not be followed in a single well.

On the other side, in situ analyses required troughs of several milliliters to ensure flat and large surfaces. Direct imaging of the lipidic surfaces was performed by Brewster angle microscopy or by epifluorescence microscopy with fluorescent lipids. The binding of proteins was analysed by epifluorescence, ellipsometry, shearing force (Venien-Bryan et al., 1998), surface pressure measurements (Ellis et al., 1997; Mosser and Brisson, 1991b) and the 2D crystallization by X-ray grazing (Lenne et al., 2000). Results obtained from these methods have provided insights into the formation of 2D crystals at the surface that includes the steps of binding to the surface, free diffusion of lipid/protein complexes favoring protein/protein interactions and 2D crystallization. However, the studies were restricted to few proteins like streptavidin available in very large amount.

Here we report the use of reflected light microscopy (RLM) for the in situ screening of events occurring at the surface; i.e. the formation of the lipid layer, the binding of soluble or micellar membrane proteins and the reconstitution of protein containing bilayers. This significantly reduces the lipid layer trials and the observations in transmission electron microscopy. Finally, this provides new insights on the mechanism of proteins binding and on 2D crystallization at the lipid layer.

2. Materials and methods

Lipids including dioleoylphosphatidylcholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) (Ni^{++}-NTA DOGS), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), egg phosphatidylcholine (EPC), egg phosphatidic acid (EPA), were from Avanti Polar Lipids. Gb3, globotriaosylceramide was purchased from Matreya LLC, USA, and rhodamine-DHPE from Invitrogen. His_6-BmrA was expressed in *Escherichia coli* and purified in *n*-dodecyl-β-D-maltoside (DDM) according to (Steinfels et al., 2004). Shiga toxin B subunit (StxB) was a generous gift of Dr. L. Joahnnes (Institut Curie).

All experiments were performed with Teflon wells of 4 mm diameter, 60 µl volume with a side hole to allow the addition of proteins, detergents or Bio Beads (Levy et al., 1999). Lipid layer at the air/water interface were obtained by spreading 0.5 µl drop of lipids solubilized in $CHCl_3$ or a mixture of $CHCl_3/CH_3OH$ (9/1 v/v) at 0.5, 0.2 or 0.1 mg/ml at room temperature onto the buffer solution and let 30 min before protein incubation.

2.1. Binding of proteins

The binding and 2D crystallization of StxB were performed according to (Hagnerelle et al., 2002). Briefly, a 0.5 µl drop of DOPC/Gb3 or EPC/Gb3 (1/1 w:w) at 0.5 mg/ml was spread at room temperature on 50 mM Tris ph 7, 150 mM NaCl buffer before injecting StxB at 50 µg/ml. The binding of purple membranes was performed by spreading 0.5 µl of 0.2 mg/ml of positively charged lipid layer of DOTAP, followed by incubation of purple membranes at 10 µg/ml in a buffer of 50 mM MES, pH 5.5, 50 mM NaCl.

2.2. Purification of His_6 BmrA onto Ni^{++}-NTA-DOGS lipid surface

Escherichia coli membranes containing His-BmrA were diluted at 1 mg/ml in 50 mM Tris, 150 mM KCl, protease inhibitor and solubilized with 1% DDM, 1 h at 4 °C. After centrifugation to remove non solubilized material, 2.5 µl of solubilized membranes were added below a previously spread lipid layer of Ni^{++}-NTA-DOGS/DOPC (1/1 w:w) at 0.1 mg/ml. The binding buffer 50 mM Tris–HCl, pH, 7.5, 150 mM KCl was supplemented with 10 mM imidazol to decrease unspecific binding of proteins onto the functionalized lipid. Before addition of solubilized membrane, DDM was added below the lipid layer at a final concentration of 0.05% i.e. 5–6 × cmc. Proteins were incubated at 4 °C upon gentle stirring for 24 h to increase the amount of bound protein, although few proteins were already present few hours after addition of solubilized membrane.

Proteins were recovered as described (Kelly et al., 2008). Briefly, a surface with bound proteins was picked up with a carbon-coated gold EM grid, carefully blotted with Whatman 4 filter paper and incubated with a 10 µl drop of the same buffer present in the well including detergent, and supplemented with 300 mM imidazole. Proteins eluted from twenty-four Ni^{++}-NTA-DOGS lipid layer were analysed by SDS–PAGE after silver staining or immunolabelling using an antibody anti-His.

2.3. 2D crystallization of BmrA

2D crystals of BmrA were obtained according to (Orelle et al., 2008). Briefly, proteins at 0.25 mg/ml in 50 mM Tris–HCl, pH 8, 5 mM, $MgCl_2$, 150 mM KCl, 5 mM benzamidine, 50 mM imidazole, and 0.5% DDM were supplemented with EPC/EPA (9/1 mol/mol) at a lipid/protein ratio 0.5–1 (w/w) and solubilized for 1 h at room temperature. This mixture was added with a 10-fold dilution below a lipid layer of Ni^{++}-NTA-DOGS and DOPC (1/1 w/w) spread at 0.1 mg/ml. The DDM and protein concentrations of 0.05% and 25 µg/ml, respectively, ensured the solubility of proteins in micelles of detergent. The reconstitution well contained 50 mM Tris–HCl, pH 8, 5 mM $MgCl_2$, 150 mM KCl, 5 mM benzamidine, and 20 mM imidazole. BmrA was bound in post-hydrolytic state in presence of 5 mM ATP plus 1.5 mM orthovanadate. After overnight binding of the His-tagged protein to the surface, detergent was removed by addition of 2 mg of Bio-Beads, leading to reconstitution after 2 days at 4 °C.

In all experiments, proteins were present at the surface only when Ni^{++}-NTA-DOGS lipid was present in the composition of the monolayer, and no proteins were found at imidazole concentration above 50 mM or EDTA above 5 mM. Proteins were found at the surface of Ni^{++}-NTA-DOGS lipid above 5% mol/mol and with DOPC or DMPC as diluent lipid. As already reported by us and others (Arechaga and Fotiadis, 2007; Lee et al., 2008; Levy et al., 2001; Levy et al., 1999), it was important to spread the lipid layer before the addition of solubilized protein to stabilize the lipid layer in presence of detergent.