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The 2DX robot: A membrane protein 2D crystallization Swiss Army knife

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ABSTRACT

Among the state-of-the-art techniques that provide experimental information at atomic scale for membrane proteins, electron crystallography, atomic force microscopy and solid state NMR make use of two-dimensional crystals. We present a cyclodextrin-driven method for detergent removal implemented in a fully automated robot. The kinetics of the reconstitution processes is precisely controlled, because the detergent complexation by cyclodextrin is of stoichiometric nature. The method requires smaller volumes and lower protein concentrations than established 2D crystallization methods, making it possible to explore more conditions with the same amount of protein. The method yielded highly ordered 2D crystals diffracting to high resolution from the pore-forming toxin *Aeromonas hydrophila* aerolysin (2.9 Å), the plant aquaporin SoPIP2;1 (3.1 Å) and the human aquaporin-8 (hAQP8; 3.3 Å). This new method outperforms traditional 2D crystallization approaches in terms of accuracy, flexibility, throughput, and allows the usage of detergents having low critical micelle concentration (CMC), which stabilize the structure of membrane proteins in solution.

1. Introduction

The atomic structure of a membrane protein is a prerequisite for understanding its function. The recent structural genomics initiatives and the related rapid method development in X-ray crystallography have lead to a massive increase of experimentally solved structures, namely from 500 in 1990 to 55,000 today (http://www.rcsb.org/pdb/statistics/contentGrowthChart.do?content=total&seqid=100). However, the number of membrane protein structures is lagging behind: as of today less than 200 unique membrane protein structures are available (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Moreover, proteins in 3D crystals are usually not in their native lipidic environment, which may prevent their native oligomeric state or conformations. Electron crystallography performed in a TEM (Transmission Electron Microscope) is an established technique for high-resolution structure determination, provided that large and highly ordered 2D crystals of membrane proteins are available (Fujiyoshi, 1998; Henderson et al., 1986). Reconstituting the solubilized membrane protein in the presence of lipids under tightly controlled conditions allows such crystals to be produced. Similar to 3D crystallization, the high dimensionality of parameter space and our limited understanding of the influence of additives, pH, lipids, and detergents make rigorous screening of crystallization conditions a necessity. Due to their hydrophobic nature, membrane proteins have to be kept in solution with detergents during purification. Reconstitution of the protein into a lipid bilayer is usually achieved by removing the detergent from ternary mixtures consisting of detergent micelles, solubilized proteins and solubilized lipid molecules or from detergent-destabilized lipid vesicles (Paternostre et al., 1988). Several methods for detergent removal have been described, such as (i) dialysis against detergent-free buffer (Engel et al., 1992), (ii) adsorption of detergent molecules to polystyrene beads (Rigaud et al., 1997), (iii) dilution of the corresponding mixture below the critical micelle concentration (CMC) of the detergent (Dolder et al., 1996; Rémigy et al., 2003), and (iv) the use of cyclodextrins to chelate the detergent in solution (Signorell et al., 2007). Dialysis is the most frequently used method to reconstitute proteins in a lipid bilayer and to produce 2D crystals. A dialysis machine providing a stable crystallization environment (buffer and temperature) for 30 samples at a time has been developed (Ringler et al., 2000). Recently, a dialysis block compatible with classical 96-well plates has been presented (Vink et al., 2007). For detergents such as octyl-β-D-glucopyranoside (OG), the CMC can be reached by dialysis within a day, whereas a long dialysis time is needed to remove low CMC detergents. Therefore, the dialysis method is only practical for medium to high CMC detergents

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(typically CMC > 1 mM). Furthermore, the detergent removal rate cannot be accurately controlled with this method. Polystyrene beads (Biobeads) can accelerate detergent removal considerably, because they efficiently capture detergents in hydrophobic pores without removing the lipids (Rigaud et al., 1997). This method is more efficient than dialysis when the protein is solubilized in mixtures of low CMC detergents, and is the method of choice for the monolayer technique (Lévy et al., 1999). However, despite an attempt to automate this procedure (Nakata and Inoue, 1998), this method requires manual beads addition lacking the precision required for reproducible 2D crystallization in small volumes. Finally, the dilution approach allows the kinetics of detergent removal to be controlled independently of the CMC, but strongly depends on the initial detergent concentration, and leads to a decrease of the protein concentration during the experiment (Rémigy et al., 2003). In general, 2D crystallization methods require significant amounts of usually scarcely available membrane proteins, and are not compatible with automation. In pursuit of the goal to automate 2D crystallization and to reduce the sample volume for large crystallization screens, we developed a crystallization robot based on the cyclodextrin-driven detergent removal approach (Degrip et al., 1998; Signorell et al., 2007).

Cyclodextrins provide a powerful alternative to crystallize membrane proteins (Signorell et al., 2007). Cyclodextrins are cyclic oligosaccharides, composed of 5 or more α-D-glucopyranoside units, with a hydrophobic interior. They form inclusion complexes with detergents or mixture of detergents regardless of their CMCs, thereby neutralizing their solubilization effect. Large detergents such as CHAPS (3-[(3-cholamidopropyl) dimethyl-ammonio] propanesulfonic acid) are efficiently chelated by γ-cyclodextrins, while detergents with alkyl chains are efficiently neutralized by β-cyclodextrin. We used methylated β-cyclodextrin due to its higher solubility in aqueous solutions and its ability to remove all detergents used in this work. Because cyclodextrin–detergent complexes form at a specific stoichiometry (Signorell et al., 2007), detergent can be removed in a precisely controlled manner. Here we exploited the compatibility of this method with microfluidic devices to develop the first high throughput 2D crystallization (2DX) robot with full control of crystallization parameters over all wells of standard 96-well plates. The robot allows optimization of the crystallization kinetics by precise cyclodextrin dispensing, control of the crystallization volume, regulation of the sample temperature between 10 and 50 °C, and it monitors the sample homogeneity and the formation of aggregates by light scattering. Crystallization results from a β-barrel (aerolysin) and two α-helical membrane proteins (SoPIP2;1 and human AQP8, hAQP8) demonstrate the efficiency of the method. Aerolysin (Bernheimer et al., 1975; Gurcel et al., 2009) is a pore-forming toxin that contributes to the pathogenicity of *Aeromonas hydrophila.* Plant SoPIP2;1 and hAQP8 are water channel proteins playing a crucial role in cellular osmoregulation. In addition, hAQP8 was reported to be an ammonia transporter (Saparov et al., 2007).

2. Materials and methods

Methyl-β-cyclodextrin was from Fluka, lipids were from Avanti Polar Lipids and detergents were from Anatrace. The phospholipids were dissolved in chloroform (10 mg/ml), dried under a stream of argon and kept overnight in a desiccator and weighed. The lipids mixtures were then sonicated for 2 min in an aqueous solution to a final concentration ranging from 5 to 10 mg/ml.

Lipids used for the screening of aerolysin were *Escherichia coli* polar extract, Soybean polar extract, DMPC (1,2-dimirystoyl-*sn*-glycero-3-phosphocholine), DLPC (1,2-dilauryl-*sn*-glycero-3-phosphocholine), POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), POPA (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate), DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), and DOPA (1,2-dioleoyl-*sn*-glycero-3-phosphate). The lipid to protein ratio (LPR) ranged from 0.2 to 1.4 (w/w) in most cases, with high LPRs of 5–10 (w/w) applied for initial tests. Soybean and *E. coli* polar extracts at LPRs ranging from 0.1 to 0.6 (w/w) were used for SoPIP2;1 and DOPA/DOPE/DOPC (at a ratio of 1:2:7) as well as POPE/POPC (at a ratio of 3:7) at LPRs ranging from 0.2 to 0.8 (w/w) were used for hAQP8 crystallization.

2.1. Detergent/cyclodextrin titration

During purification, as the solubilized protein passes through ion-exchange or affinity columns or undergoes concentration by centrifugation, the detergent in the mixture gets diluted or concentrated. To master crystallization kinetics, detergent concentration after protein purification was systematically measured by the "DropBox" method (Kaufmann et al., 2006). The DropBox measures the detergent concentration based on the contact angle made by a sample droplet on a hydrophobic surface. The observed angle is compared with a calibration curve for the particular detergent to determine its concentration. The amount of cyclodextrin required to neutralize the detergent was then added according to detergent/cyclodextrin titration curves (Signorell et al., 2007). When a detergent calibration or a detergent/cyclodextrin titration could not be performed, i.e. a mixture of detergents has been used to purify the protein, a second method was applied: a small amount of detergent solubilized protein was mixed with cyclodextrin solutions of different concentration. The calibration curve of a cyclodextrin solution displays a plateau at around 90° as measured by the DropBox. When the cyclodextrin amount is insufficient to neutralize the detergent, the surface tension decreases and the contact angle is lower than 90°. When high CMC detergents are used it is important to ensure that the raise of the contact angle beyond 90° is due to the cyclodextrin neutralization and not the dilution effect. In such case, underestimation of the cyclodextrin to be added during reconstitution will lead to incomplete detergent removal.

2.2. Robot design

The robot (Fig. 1) is controlled over a GUI (graphical user interface) hosted on a Linux computer. The main window is divided into two panels: the "*experiments layout*" where all relevant data required to drive the experiment are entered, and the "*experiments monitoring*" where all experimental details can be monitored. In the "*experiments layout*" the user edits parameters to conduct the experiment: initial volume, cyclodextrin to add, temperature profile, shaking period and frequency, and constraints. Constraints define threshold values upon which to change an experimental parameter, i.e. sample volume or cyclodextrin addition rate for a set of wells. Threshold values include elapsed time, total amount of added cyclodextrin and light scattering intensity. Up to 10 constraints can be added for one experiment and each experiment is independent from the other. Wells can be addressed separately or in groups. Shaking period, frequency and temperature profile can be changed dynamically during the run. Through the GUI users can load or unload the plate at anytime to take aliquots in order to follow the experiments or to add a new experiment. After sample removal, the robot will fill the wells to the previous volume automatically, allowing experiments to continue. All parameters can be saved in or loaded from a data file. The "*experiments monitoring*" tab contains all the information of the experiments: real temperature profile, light scattering intensity profile, total volume per well

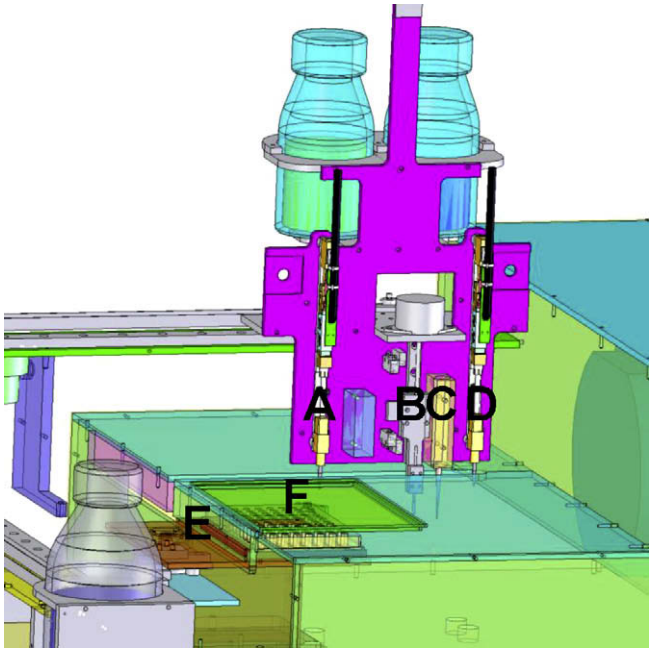

Fig. 1. Membrane protein crystallization robot closeup. (A) Contact less cyclodextrin dispenser. (B) Contact less volume measurement sensor. (C) Light scattering detector. (D) Contact less water dispenser maintaining the targeted sample volume. Homogenization of the crystallization mixture is made by (E) an orbital microplate shaker allowing for simultaneous light scattering measurement. (F) 96 wells commercial transparent microplate. Temperature is regulated by a Peltier device.

and added cyclodextrin (real kinetic profile). This information is displayed continuously in the graphical user interface and saved in a log file for further inspection. Crystallization mixtures containing detergents exhibit enhanced wetting behavior. By using contact-less dispensers, cross-contamination is prevented. The dispensers (Seyonic SA, Neuchâtel, Switzerland) enable contactless addition of cyclodextrin and water down to the nanoliter level. Feedback tests to control the dispensed volume based on a flow sensor embedded in each pipette were implemented to ensure high reliability during reconstitution. Software provided by Seyonic is used to prime and purge the system and to regulate pipettes pressure provided by pressurized high grade CO_2 and Helium. The cyclodextrin solution stock is made of 0.02% NaN_3, 5% methyl-β-cyclodextrin and bi-distilled water. The water stock is made of bi-distilled water only. Both solutions are filtered using a 0.2 μm filter. When the cyclodextrin and water solutions are filtered and sparged with helium gas, the dispensers flushed with CO_2 and the system properly primed, it is stable for weeks. A contactless sensor made of a capacitive sensor actuated by a stepper motor measures the sample volume without inter-well contamination. In combination with the water dispenser the sample volume is adjusted to a target value with a precision better than 2 μl.

A similar static light scattering system as in Rémigy et al. (2003) has been integrated into the 2DX robot. Every well passes below a 650 nm/1 mW laser once per cycle and is illuminated for 300 ms. In case a light sensitive protein is investigated the laser can be turned off.

The duration of one cycle comprising cyclodextrin addition, volume measurement, light scattering measurement, water addition, and shaking depends on the number of wells occupied and the temperature. At 37 °C and 96 samples to monitor, one cycle takes less than 15 min. The maximum cyclodextrin volume addition is around 1250 nl per cycle per well when the plate is fully loaded. Typical cyclodextrin addition ranges from 50 to 2000 nl/h per well. The evaporation rate per well is approximately 1 μl per 20 min at 23 °C and 2–3 μl per 20 min at 37 °C depending on the ternary mixture and the air humidity. Since both the water pipette as well as the cyclodextrin pipette are "in-line dispensers", enough water can be added to preserve the original volume for the duration of the experiment.

2.3. Ternary mixture preparation

We generally set the pH of the protein samples during the last purification step (usually an elution). When this was not feasible, a fast dialysis was performed to adjust pH. The protein is mixed with the aqueous lipid suspension within the 96-well plates. Screening additives (salts, buffers, ligands) can be added manually as well as by using a pipetting robot.

2.4. Production and purification of aerolysin

Production and purification of pro-aerolysin was performed as previously described (Buckley, 1990). *Aeromonas* strain CB3–pNB5 carrying the plasmid for WT over-expression were grown overnight in LB medium (Davis et al., 1980) supplemented with 0.2% glucose at 26°. The pre-culture was then diluted 1:50 and allowed to grow to OD = 0.6–0.8 with vigorous shaking followed by IPTG addition to induce protein production. The culture grew for ~12 h and the bacteria were discarded by centrifugation. The proteins secreted by the bacteria into the medium were precipitated by addition of 60% ammonium sulfate (final concentration) and resuspended in 20 mM phosphate buffer, 300 mM NaCl, pH 6. The protein sample was further dialyzed against 2× 2 L of the same buffer and a first round of purification was performed by ion exchange chromatography using a hydroxy-apatite column. The bound protein was eluted using a gradient (20–150 mM phosphate buffer, 300 mM NaCl, pH 6). The fractions collected were checked for purity and the fractions containing the pro-aerolysin were pooled and dialyzed against 20 mM Tris, 100 mM NaCl, pH 7.4. A new round of purification using a DEAE column and an elution gradient ranging from 0 to 500 mM NaCl, 20 mM Tris, pH 7.4 yielded highly pure protein with concentrations ranging from 0.2 to 6 mg/ml. The protein concentration was estimated using UV-spectroscopy as previously described (Buckley, 1990). The purity of the sample was checked by SDS–PAGE and the activity of the toxin was assessed by hemolysis.

For crystallization trials aliquots of highly pure heptamerized protein having a concentration above 1 mg/ml were used. The bacteria produce the protein as an inactive pro-toxin (Bernheimer et al., 1975; van der Goot et al., 1992). To have a minimal loss of protein the activation of the toxin and the removal of the pro-peptide were performed using trypsin immobilized on agarose beads at pH 8.5 to prevent the heptamerization (van der Goot et al., 1992). After addition of the trypsin, the sample was incubated on a rotary shaker at 4 °C for few hours prior to the removal of the agarose beads by centrifugation. This step results in complete activation of the toxin with minimal heptamerization. To induce oligomerization the activated toxin sample was dialyzed against 10 mM Hepes, 10 mM NaCl, pH 7.4, using a dialysis membrane having a cut-off of 3.5 kDa at 4 °C.

One percent of detergent (OG, Octyl-POE or octyl-pentaoxylene (C8E5)) was added to the activated aerolysin stock obtained as described above and the mixture was incubated at room temperature 30 min to allow heptamerization of the protein. To screen crystallization conditions, the purified protein was supplemented with buffer stock solutions or dialyzed against the desired buffers. Ternary mixtures of 30–50 μl were used for screening for crystallization conditions. Best results were obtained with C8E5 detergent using either POPA:POPE:POPC (1:2:7) or DOPA:DOPE:DOPC (1:2:7) lipid mixtures with LPRs between 0.8 and 1.2 (w/w). Various buffers ranging from pH 5.4 to pH 8 and containing NaCl with

concentration ranging from 100 mM to 1 M were tested with best results at pH 5.4 and with 1 M NaCl.

2.5. Purification of SoPIP2;1 S115A in DDM and OG

The spinach plasma membrane intrinsic protein SoPIP2;1 S115A mutant was heterologously expressed in the methylotrophic yeast *Pichia pastoris* as described for the wild type protein (Karlsson et al., 2003). Pre-washed membranes (Hasler et al., 1998) were mixed with two different solubilization buffers (10 mM phosphate buffer, pH 7.8, 10% glycerol, 12% OG or 10 mM phosphate buffer, pH 7, 10% glycerol, 3% DDM) to an end-concentration of 6% for OG and 1.5% DDM and solubilized at room temperature for 1 h. Unsolubilized material was pelleted at 20,000g, 4 °C for 40 min. The supernatant was mixed 1:1 (v/v) with equilibration buffer (10 mM phosphate buffer, pH 7.8, 1% OG or 10 mM phosphate buffer, pH 7, 0.1% DDM) prior to loading onto a CM-Sepharose column that was pre-washed with approximately 15× bed volumes of 1 M NaCl and equilibration buffer. The protein was loaded on the column, washed with 4 ml of equilibration buffer and eluted with elution buffer (10 mM phosphate buffer, pH 7.8, 200 mM NaCl, 1% OG or 10 mM phosphate buffer, pH 7, 250 mM NaCl, 0.1% DDM). The protein concentration was measured by UV-spectroscopy at 280 nm (Gill and von Hippel, 1989).

Before mixing purified SoPIP2;1 S115A with lipids, the protein was dialyzed for 2 h to adjust the salt concentration and the pH according to the crystallization conditions used. The cyclodextrin addition rate was chosen to have DDM fully neutralized after 40 h. The total ternary mixture volume ranged from 20 to 40 μl and was kept constant during reconstitution. The NaCl concentration ranged from 100 to 500 mM, $MgCl_2$ from 40 to 60 mM, constant temperature during the experiments ranged from 23 to 37 °C, and protein concentration from 0.5 to 1 mg/ml. Invariant parameters: cyclodextrin addition rate was 7.5–15 nl/(h μl), crystallization buffer was 20 mM Tris–HCl, pH 8, 2 mM DTT, LPR ranged from 0.1 to 0.6 (w/w). Best results were obtained with LPR 0.5 ± 0.05 (w/w).

Fifteen microliters of a ternary mixture, containing lipids, protein (~1 mg/ml) and detergent were placed in a well. In addition, 5 μl of adjustment buffer were used to change the concentration of additives. If the concentration of the purified protein was high enough (>2 mg/ml), the ratio of ternary mixture to adjustment buffer was changed to 1:1 (V/V) to lower the absolute error. For lower protein concentrations (<0.5 mg/ml), the ratio could be kept at 1:1 and in addition, the sample was concentrated at the beginning of the crystallization experiment, i.e. by setting the initial volume to 40 μl and then letting the sample dry to 20 μl. Hence 1 mg of SoPIP2;1 S115A is sufficient to test 15 conditions, each at LPR 0.1, 0.2, 0.3, 0.4 and 0.5 (w/w), respectively, allowing rapid initial screening of 75 crystallization conditions. For comparison, the same amount could be used to screen 10–15 conditions by dialysis.

2.6. Purification of hAQP8 in DDM

Membranes from *P. pastoris* heterologously expressing His-tagged hAQP8 (Maria Fellert, unpublished results) were solubilized at a 1:1 ratio with purification buffer (20 mM Hepes, 300 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol (β-ME), 0.03% NaN_3, *Complete Protease Inhibitor – Roche*, pH 7.8) containing 4% of DDM at room temperature for 3 h. Unsolubilized material was pelleted at 150,000g, 4 °C for 40 min. The supernatant was mixed overnight at 4°C with Ni–NTA agarose (Qiagen) after addition of 15 mM imidazole. Purification was performed on PolyPrep columns (Bio-Rad) at 4 °C. Ni–NTA agarose was washed on the column with 50 bed volumes of purification buffer with 0.04% DDM and 75 mM imidazole. The protein was eluted in purification buffer containing 0.04% DDM and 300 mM imidazole. The protein sample concentration was measured by UV-spectroscopy at 280 nm (Gill and von Hippel, 1989). The detergent concentration was also measured with the DropBox (Kaufmann et al., 2006).

Purified protein at a concentration of 1 mg/ml was dialyzed against crystallization buffer (20 mM Hepes, 100 mM NaCl, 5 mM EDTA, 10% glycerol, 2 mM DTT, 0.03% NaN_3, pH 7) for 3 h at 4 °C. The protein was then mixed with selected lipids to final LPRs ranging from 0.2 to 0.8 (w/w). The total ternary mixture volume was 30 μl. Detergent concentration was 0.15% DDM and the cyclodextrin titration rate ranged from 5 to 20 nl/(h μl). The 2D crystallization process was monitored by light scattering intensity measurement. The following temperature profile was selected: 12 h at 23 °C, 12 h increasing linearly to 37 °C, 48 h at 37 °C. When proteins formed sheets and large vesicles, 0.3 μg of endoprotease Lys-C (Roche) was added. Removing the His-tag improved the crystal quality. Endoprotease Lys-C hydrolyzes peptide bonds at the carboxyl side of lysines. In the 238-amino-acid hAQP8 construct, seven possible Lys-C cleavage sites were found. However, it should be noted that when the protein is embedded in a lipid bilayer only three lysines are exposed to the enzyme at the C- and N-termini. Therefore the His-tag at the C-terminus as well as 10 aa at the N-terminus are cleaved without disruption of the protein, as confirmed by mass spectrometry and SDS–PAGE/Western blot analysis (results not shown).

2.7. Transmission electron microscopy (TEM) and cryo-electron microscopy (cryo-TEM)

For imaging of negatively stained samples, aliquots of 5 μl were adsorbed on the carbon-film coated copper grids, washed with 10 droplets of pure water and subsequently stained with 2% uranyl-acetate. Images were recorded using a Philips CM100 TEM operated at 100 kV. Electron diffraction patterns were recorded using a Philips CM200FEG equipped with a Gatan (Gatan, Warrendale, PA) cryo-stage. Aerolysin samples were adsorbed onto holey carbon film grids (Quantifoil, Germany) rendered hydrophilic by glow discharge at low pressure in air, and then plunged in liquid ethane cooled at −176 °C (Dubochet et al., 1988). Aquaporin samples were adsorbed on Molybdenum grids coated with a carbon film and embedded in 7% trehalose using the back-injection technique (Hirai et al., 1999), and frozen in liquid nitrogen. Alternatively, 2D crystals were adsorbed to Quantifoil grids (Micro Tools GmbH, Jena, Germany) and vitrified in liquid ethane. Diffraction patterns were recorded at liquid nitrogen temperature on a Gatan UltraScan 2k × 2k CCD camera using at a dose of <5 electrons/$Å^2$).

3. Results and discussion

3.1. Crystallization strategy

After a purification protocol for a membrane protein has been established, a strategy for crystallization conditions should be devised. The large parameter space that needs to be sampled (buffers, additives, lipids, detergents, temperature, kinetics) can be divided in several initial screens. Buffers, lipids and an adequate LPR range should be first explored. When performing reconstitution using cyclodextrin, the kinetics of crystallization is independent from the detergent nature, hence the choice of the detergent for reconstitution is solely dictated by the stability of the protein during purification. After the detergent concentration has been measured to determine the amount of cyclodextrin needed to induce reconstitution, an approach comparable to methods employed by 3D crystallographers is used: standard screens solutions are prepared excluding conditions known to inactivate or denature the protein. As an example, using buffer and lipid stock solutions one can

screen six different buffers, four lipids and four different LPRs with as little as about 1 mg of protein. In this initial screen kinetics and temperature should be set depending on the stability of the protein. In general, gradual detergent removal over 40–50 h at room temperature gave the best results for an initial screen, whereas a rapid (6 h) detergent removal can be performed to quickly identify conditions under which the protein reconstitutes in small lipid vesicles. Once these conditions are identified the kinetics can be adjusted to obtain large vesicles and sheets. Hence, testing one thousand conditions may take three weeks, given that an automated solution for grid preparation (negative staining of the sample) and screening would be available.

Because temperature variations are known to affect the phase behavior of lipid–detergent mixtures (Sennoga et al., 2003) and the fluidity of the lipid bilayer, temperature controlled crystallization devices have been build for both 2D (Jap et al., 1992; Rémigy et al., 2003) and 3D crystallization of proteins (Berg et al., 2002). Our 2DX robot has a controlled environment providing stable temperature distribution across the crystallization plate. For the proteins studied here, the initial stage of reconstitution was best achieved at room temperature, while the final detergent removal was at a temperature between 30 and 40 °C for obtaining highly ordered sheets. Similar conditions were previously found to yield coherent arrays of AQP2 (Schenk et al., 2005) or AQP1 (Walz et al., 1994).

3.2. Aerolysin crystallization

Early attempts to crystallize aerolysin yielded 2D crystals that provided the original model of the membrane inserted pore (Wilmsen et al., 1992). Starting with these initial crystallization conditions, we strived for improving the crystals by systematic variation of all parameters. The 2DX robot being able to handle small crystallization volumes (down to 10 µl) and up to 96 conditions per run allowed us to efficiently vary selective parameters in small increments. Small packed aerolysin arrays diffracting to 20–25 Å obtained initially were improved to large highly regular crystals that diffract to 3 Å resolution (highest observable Friedel pairs) (Fig. 2). While aerolysin easily packs into semi-ordered arrays, it forms large highly coherent crystals only under very specific conditions. In a first step, we identified the ideal detergent/lipid mixture by testing a wide range of lipids and detergents. In the second step, the buffer pH and the salt concentration were optimized. Interestingly, the detergent/lipid mixture giving the best results could be used over a large range of LPRs. LPRs of up to 10, w/w resulted in highly crystalline patches surrounded by empty lipids, suggesting a minor importance of the LPR for aerolysin crystallization. This phenomenon has subsequently been observed as well during crystallization of aerolysin by dialysis.

As reported previously, the detergent removal rate has a significant influence on the quality and size of 2D crystals for any membrane protein (Dolder et al., 1996; Rémigy et al., 2003; Signorell et al., 2007). The rate should be fast to stabilize the protein in the lipid environment, but sufficiently slow to allow proteins to pack regularly in the bilayer. We therefore explored the influence of detergent removal rates for all three membrane proteins studied here. Since this rate can be set for each well independently, multiple kinetics experiments were run simultaneously, regardless of the nature of detergent. Such rates could only be achieved until now by the Biobeads method but without the efficiency and precision provided by the 2DX robot. As illustrated in Fig. 2 aerolysin

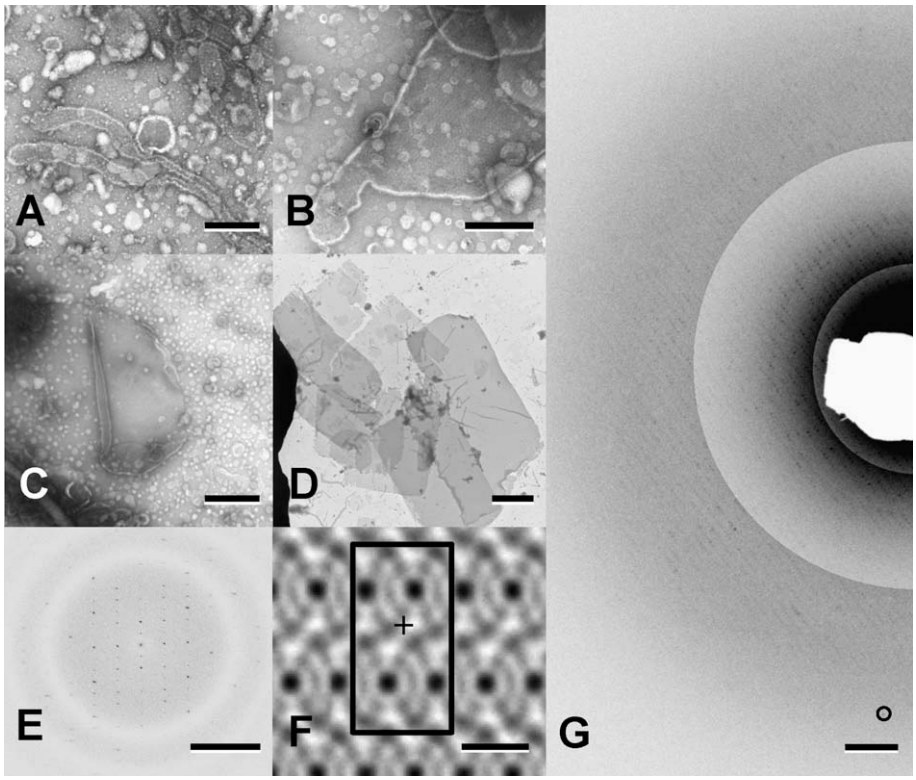

Fig. 2. The influence of reconstitution kinetics on the 2D crystallization of aerolysin. For each case shown the amount of cyclodextrin added was identical and sufficient to neutralize all the detergent present in the initial ternary mixture, but the rate was different. Samples were negatively stained and examined by transmission electron microscopy. Full complexation of the detergent was reached after 6 h for (A), 12 h for (B), 24 h for (C), and 48 h for (D). Scale bars are 100 nm for (A) and (B), 500 nm for (C), and 1 µm for (D). (E) The calculated diffraction pattern from a negatively stained crystal such as shown in (D) exhibits orders to 25 Å resolution. Scale bar is 5 nm^{-1}. (F) Aerolysin unit cell (a = 148 Å, b = 282 Å, α = 90°) calculated from (D) reveals the morphology of the membrane embedded toxin. Scale bar is 10 nm. (G) Cryo-electron diffraction of crystals displayed in (D) adsorbed on an holey carbon grid (Quantifoil, Jena, Germany) and vitrified in liquid ethane. The spot marked by a circle corresponds to a resolution of 2.9 Å. Scale bar is 2 nm^{-1}.

proteoliposomes having diameters smaller than 200 nm were obtained by fast detergent removal (within 6 h, Fig. 2A), but their size significantly increased when titration was slowed down. Vesicles (diameter 300 nm, Fig. 2B) were found when detergent removal was completed after 12 h, and after 24 h small sheets were observed (Fig. 2C). Two-dimensional crystals reached their maximum size when detergent removal was achieved over 48 h or more, with both C8E5 and DDM (Fig. 2D). While the aerolysin was allowed to reconstitute at 23 °C during the initial detergent removal process, the temperatures was elevated to 30 °C or more to obtain highly ordered large crystalline sheets.

3.3. SoPIP2;1 S115A crystallization

The plant aquaporin SoPIP2;1 is known to crystallize both in 2D (Kukulski et al., 2005) and 3D (Tornroth-Horsefield et al., 2006; Nyblom et al., 2009). By dialysis, this mutant crystallized under the same conditions as the wild type to form highly ordered 2D crystals using OG (Kukulski, unpublished results). To demonstrate the ability of the 2DX robot to crystallize membrane proteins solubilized in low CMC detergents, we explored the 2D crystallization of the mutant purified in dodecyl-β,D-maltoside (DDM). Initial crystallization trials revealed that DDM strongly affected the crystallization conditions. The 2DX robot enabled us to broaden the range of screening parameters needed to rapidly obtain high quality crystals (Fig. 3). The apparent LPR at which best crystals were obtained was higher than reported previously (Kukulski et al., 2005). As the ability of detergents to co-solubilize lipids from the membranes in which the protein was expressed differs largely, the difference in LPR needed for crystallization is not surprising. Interestingly, at 100 mM NaCl, the DDM-solubilized SoPIP2;1 S115A did not arrange in regular arrays as the OG-solubilized protein does under this condition. However, at higher NaCl concentration (500 mM) the DDM solubilized protein yielded highly ordered double layered crystals showing diffraction spots beyond 3 Å (Fig. 3B) when cyclodextrin was added over 40 h to a final concentration that ensured complete neutralization of DDM. Best results were obtained when the temperature was linearly increased over 5 h from room temperature to 37 °C after 15 h. Adding the same amount of cyclodextrin over 120 h did not further improve the crystallinity of SoPIP2;1 crystals.

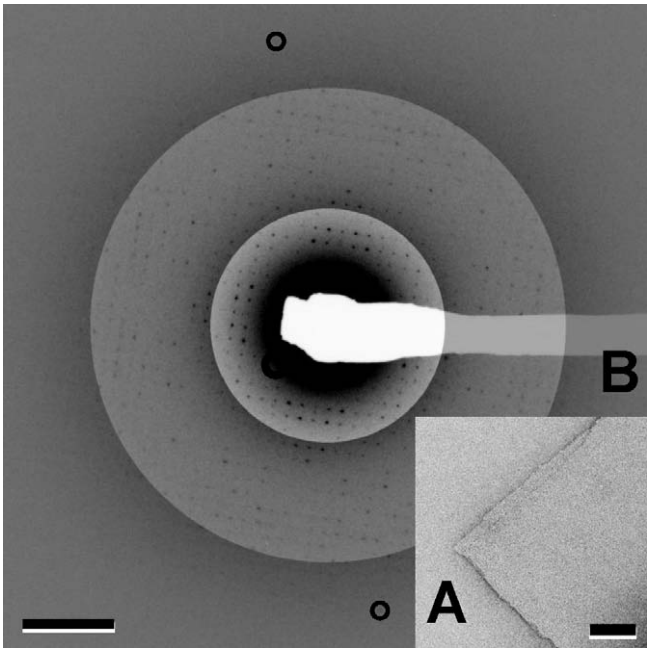

Fig. 3. SoPIP2;1 solubilized in DDM was reconstituted by adding cyclodextrin over 40 h to a final concentration that ensured complete neutralization of DDM. The inset (A) displays a negatively stained double layered SoPIP2;1 sheet. Scale bar is 100 nm. (B) Cryo-electron diffraction of trehalose embedded crystals revealed their excellent order. Scale bar is 1 nm^{-1}. The spots marked by circles correspond to a resolution of 3.1 Å.

3.4. hAQP8 crystallization

The crystallization conditions of the human aquaporin hAQP8 solubilized in DDM were screened using the 2DX robot (Fig. 4). hAQP8 is a human aquaporin of interest, being not only an efficient water channel but also a highly selective diffusion pore for ammonia (Saparov et al., 2007). hAQP8 was reconstituted as double-layered crystalline sheets and packed vesicles of a few micrometers in diameter. As illustrated in Fig. 4, we studied how the 2D crystals developed during gradual detergent neutralization by cyclodextrin from the ternary protein–lipid–detergent mixture over 60 h. The reconstitution process was monitored by light scattering (Fig. 4F), and by taking samples after 5, 11, 21 and 26 h (Fig. 4A, B, C and D, respectively), demonstrating how the crystallization kinetics can be controlled. To explore the effect of the reconstitution kinetics with hAQP8, parallel experiments were run over 60 h, while the detergent removal rates were varied from 1 to 4 folds (5, 10, 15 and 20 nl/(h μl)). When the 36 mM cyclodextrin solution was added at a rate of 20 nl/(h μl) to the crystallization mixture containing 3 mM detergent (DDM) and hAQP8 at a concentration of 1 mg/ml, small proteoliposomes were formed. In contrast, large sheets were detected during reconstitutions at a rate of 5 nl/(h μl) (Fig. 4E). Moreover, the effect of removing the 6xHis-tag of hAQP8 with the protease Lys-C was explored at different stages of hAQP8 reconstitution. Lys-C, which hydrolyzes peptide bonds at the carboxyl side of lysines, was added to improve the crystallinity of giant vesicles that formed when the reconstitution process was well advanced (Fig. 4D). If the protease was added too early aggregation occurred. Best results with DDM solubilized hAQP8 were obtained upon cleavage of the C- and N-terminal moiety, and diffraction patterns exhibited spots beyond 4 Å (highest observable Friedel pairs) (Fig. 4G). The temperature profile (12 h at 23 °C, followed by a slope reaching a plateau at 37 °C 12 h later) was key to obtaining highly ordered hAQP8 sheets.

3.5. Controlled crystallization and high throughput

To achieve a homogeneous distribution of all constituents in the crystallization mixtures, a shaker has been implemented. All membrane proteins presented in this work where shaken during crystallization, which did not prevent high quality crystals to form. We monitor the homogeneity of the solution using a static light scattering detection system (Rémigy et al., 2003), and follow the effect of detergent neutralization by cyclodextrin. The light scattering signal is displayed by the 2DX robot user interface. It indicates when components of the ternary mixture start to assemble (Fig. 4F), and can be used to regulate the cyclodextrin addition rate.

Throughout the crystallization screens samples were analyzed by negative staining in the TEM. Remaining cyclodextrin alters the contrast and was thus removed by extensive washing of the grids before staining. Large crystals were examined at cryogenic temperature after either vitrification or embedding in trehalose, and electron diffraction patterns were acquired at low dose to assess the crystallinity.

All traditional 2D crystallization methods bear limitations with respect to the amount of protein required for crystallization screens, to the reproducibility of detergent removal kinetics and especially to the feasibility of a high throughput implementation. The cyclodextrin method for quantitative detergent removal lends

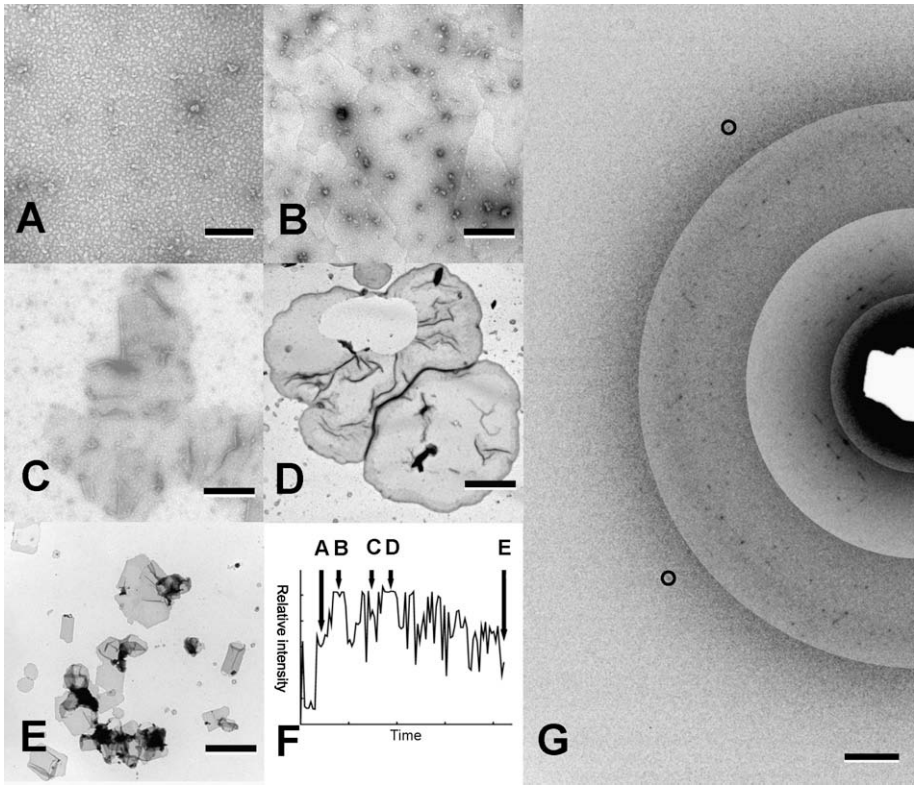

Fig. 4. Electron micrographs of negatively stained samples taken at different stages during crystallization of hAQP8. (A) After 5 h, sufficient cyclodextrin has been added to neutralize all the free detergent molecules and small proteoliposomes start to assemble. (B) After 11 h, sheets and vesicles coexist. (C) After 21 h, sheets are more numerous while the number of vesicles has decreased. (D) After 26 h giant vesicular structures are found, but their morphology does not indicate crystallinity. (E) After addition of more cyclodextrin and Lys-C endoprotease, crystalline sheets are observed at 59 h. Scale bar is 200 nm for (A), 400 nm for (B) and (C), 800 nm for (D), and 3 μm for (E). (F) Light-scattering profile monitoring the progress in reconstitution. Samples for micrographs shown in (A)–(E) have been taken at time points indicated by arrows. (G) Cryo-electron diffraction of crystals shown in (E). The spot marked by a circle corresponds to a resolution of 3.3 Å. Scale bar is 2 nm^{-1}.

itself to automation, and allows the 2D crystallization process to be fully controlled, better understood and optimized. Attempts toward this goal have been performed in the past (Dolder et al., 1996; Rigaud et al., 1997; Rémigy et al., 2003), but none of them provided such a tight control over crucial crystallization parameters (kinetics, homogenization, light scattering, temperature, and crystallization volume) as the 2DX robot described here.

Using standardized commercially available 96-well plates enabled us to upscale the screening process. Traditionally, a large screen comprised about 30–50 samples. Dialysis, the most widely used method, requires an equal amount of buttons to be prepared together with dialysis membranes and O-rings. The dialysis block (Vink et al., 2007) having the normalized 96-well plates form factor circumvents this drawback, and for each condition, a separate dialysate container is required. In dialysis the usual sample volume is between 60 and 100 μl with a typically 10^4 times larger dialysis buffer volume for detergent removal. In contrast, the 2DX robot (Fig. 1) can handle concurrently 96 samples with volumes ranging from 10 to 80 μl, with no need to exchange the buffer. Often, the addition of high affinity ligands (Scott et al., 2004) (e.g., inhibitors) or additives (Salom et al., 2006) stabilize the protein structure. Since the cyclodextrin addition is performed in batch, co-crystallization using ligands or any other small molecules is feasible. Dialysis driven reconstitution experiments are carried out with a typical dialysis membrane having a cut-off around 10–14 kDa. Such small compounds would need to be added to the dialysate.

Size and quality of the assembled protein–lipid aggregates are directly related to the detergent removal rate (Signorell et al., 2007; Lévy et al., 1990). With the dialysis method, increasing the temperature can accelerate diffusion of detergent molecules across the membrane. Thus, the crystallization velocity depends both on the CMC of the detergent as well as the temperature. In the 2DX robot, the detergent removal rate is given by the rate of cyclodextrin addition and does not depend on the temperature, therefore the temperature and the detergent removal rate can both be adjusted independently and adapted to the requirements of the protein to be crystallized. This is most useful for membrane proteins that are unstable when solubilized. The temperature also needs to be above the phase transition temperature of the lipids used when reconstitution begins. Although frequently used lipids have phase transition temperatures below 25 °C, raising the temperature to 37 °C at a later stage of the reconstitution was found to improve the crystallinity of the 2D crystals (Schenk et al., 2005; Walz et al., 1994). Here, we show that such elevated temperatures were also mandatory to obtain highly ordered crystals of aerolysin, SoPIP2;1 and hAQP8. Experiments with aerolysin and hAQP8 illustrate the decisive effect of the detergent removal rate on proteoliposomes size (Figs. 2 and 4).

Shaking has generally been excluded from traditional crystallization methods, and a "gradient" of detergent concentration was believed to improve crystallinity. In our approach, shaking enhanced reproducibility and did not affect crystallinity. Given that samples are homogeneous at the outset, static light scattering can monitor the aggregation state as it evolves during reconstitution. Scattering increases as a result of crystals or aggregates forming when the concentration of free detergent approaches the CMC, which initiates the micelle to vesicle transition. This stage was found to be the critical point during crystallization (Rigaud et al., 1995; Lambert et al., 1998). It is known that if the phase transition region is passed too quickly proteoliposomes or small vesicles will

assemble. The 2DX robot has the ability to lower the cyclodextrin addition rate when light scattering intensity reaches a given threshold. This promotes the formation of larger protein–lipid–detergent complexes, which results in larger crystals because crystallization proceeds right from this initial assembly step (Dolder et al., 1996).

The ambient liquid evaporation can be used to concentrate the sample. With a contact free level sensor, the liquid level is constantly monitored and the sample is allowed to evaporate until the desired volume is reached. Samples with a low protein concentration (<0.5 mg/ml) may benefit from the controlled evaporation feature. The method offered by the robot conveys an advantage over centrifugal concentration, because it is done in parallel with the reconstitution process, thereby reducing the time the protein remains in detergent. Also, purified membrane proteins may be lost during centrifugal concentration as they can adsorb to the cut-off membrane.

In some cases, the detergent with best protein-stabilizing properties for a given membrane protein does not solubilize the membranes efficiently. Instead of executing a detergent exchange, a step frequently detrimental to the membrane protein, one can mix detergents. Because the cyclodextrin approach is compatible with detergent mixtures, and not restricted to high CMC detergents, proteins can be extracted from the membrane with mild detergent mixtures while preserving their native and stable structure. This topic has not been investigated extensively yet but many examples showed that a mixture of two detergents was required to purify and to keep the solubilized protein stable during activity assays (Grisshammer et al., 2005; Attrill et al., 2009). The 2DX robot can neutralize both detergents at the same ratio provided that the stoichiometry of detergent complexation by cyclodextrin is comparable. Some detergents like CHAPS are known to improve stability (Cladera et al., 2004; Grisshammer et al., 2005) and may be added to the ternary mixture solution to carry out reconstitution. This sterol-based detergent has poor affinity for β-cyclodextrin but high affinity for γ-cyclodextrin probably due to its bulky hydrophobic moiety. In such a case a mixture of cyclodextrins can be used to neutralize two different detergents simultaneously. By adjusting the γ-/β-cyclodextrins ratio it would be possible to slowly complex one detergent while quickly removing the other.

4. Conclusion

The presented 2D crystallization method and the 2DX robot built to execute it confer major advantages over other methods. Smaller protein amounts are needed to perform reconstitutions, precise control of the detergent removal rate is possible as a results of the stoichiometric association of cyclodextrins with detergent molecules, and parallel reconstitution can be done in industrial standardized well plates amenable to further up scaling. The system neutralizes detergent of any nature (low or high CMCs) in a quantitative manner, allows the reconstitution process to be closely monitored by light scattering, and provides for co-crystallization of membrane proteins with small ligands. Also, turbidimetry, fluorescence spectroscopy, Raman spectroscopy, infrared/ultraviolet spectroscopy or polarization analysis can eventually be implemented. The possibility of in-line optical spectroscopic analysis will allow monitoring ligand-binding experiments consecutively to membrane protein reconstitution or liposome formation. This method represents a major step toward a completely automated approach for 2D crystallization of membrane proteins, and for the large-scale reconstitution of membrane proteins into proteoliposomes for functional tests or high-throughput ligand-binding assays. The cyclodextrin 2D crystallization robot is one element of a tool chain comprising a staining robot and a computer-controlled screening TEM for automated grid analyses. This system will allow wide screens to be executed and systematically analyzed, and will provide new possibilities to optimize the assembly of highly ordered 2D membrane protein crystals.

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