Testing Parameters for Two-Dimensional Crystallization and Electron Crystallography on Eukaryotic Membrane Proteins with Liposomes as Controls

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Membrane proteins comprise the majority of known and potential drug targets, yet have been immensely difficult to analyze at the structural level due to their location in the membrane bilayer. Removal from the membrane necessitates replacement of the phospholipid bilayer by detergents in order to maintain protein solubility. However, the absence of lipids and the presence of detergents can render non-physiological conformational changes of the membrane protein (Tate, 2006). Electron crystallography is an important method for studying membrane proteins that usually takes advantage of reconstituting the protein in a phospholipid bilayer and removal of the detergent. Richard Henderson and Nigel Unwin used this technique to elucidate the three-dimensional (3D) arrangement of the transmembrane α-helices of bacteriorhodopsin, which was the first 3D structural information on a membrane protein (Henderson and Unwin, 1975). In recent years, electron crystallography was used to study a significant number of proteins at the atomic level (Henderson *et al.*, 1990; Kühlbrandt *et al.*, 1994; Nogales *et al.*, 1998; Murata *et al.*, 2000; Ren *et al.*, 2001; Löwe *et al.*, 2001; Miyazawa *et al.*, 2003; Gonen *et al.*, 2005; Hiroaki *et al.*, 2006; Holm *et al.*, 2006).

Electron crystallography of membrane proteins involves electron cryo-microscopy (cryo-EM) and image processing techniques (Amos *et al.*, 1982) that can now fairly routinely yield high quality structural information. The main limiting factors in using this method to study membrane protein structures are two-fold: (1) the production of large, highly ordered two-dimensional (2D) crystals and (2) screening for these 2D crystals (Kühlbrandt, 1992; Schmidt-Krey, 2007). Two-dimensional crystallization of membrane proteins by dialysis is comparable to reconstitution of solubilized membrane proteins for biochemical experiments (Fig. 1), except for the objective in 2D crystallization of having the largest possible amount of protein in the phospholipid bilayer. The protein-detergent mixture is combined with a lipid-detergent mixture, which is then dialysed against detergent-free buffer. Under ideal conditions, the membrane proteins form 2D crystals in each or most of the proteoliposomes.

Screening for crystals requires careful evaluation of the specimen so as not to miss the crystals, which can initially be small, sometimes only several unit cells, and few in number. Efforts in a number of laboratories are beginning to contribute major advances towards high-throughput screening (Cheng *et al.*, 2007; Nakamura *et al.*, 2003; Vink *et al.*, 2007) and equipment for controlled, large-scale crystallization (Engel *et al.*, 1988) as well as image processing (Gipson *et al.*, 2007). Little, however, is known about the parameters that influence crystal formation. This type of information could greatly reduce the required time and effort in this crucial step towards not only obtaining structural data on membrane proteins, but would also provide invaluable information for the design of automated and large-scale trials. Yet general conclusions on 2D crystallization conditions will require the accumulation of information from a large number of individual crystallization trials. Engel and coworkers have provided a particularly sizable and helpful contribution in documenting their findings over the years (Stahlberg *et al.*, 2001; Werten *et al.*, 2002).

Crystallization conditions for two eukaryotic membrane proteins with known crystallization conditions were further tested with the long-term aim of providing additional data for generalizing 2D crystallization screening. These proteins, human vitamin K-dependent γ-glutamyl carboxylase (Schmidt-Krey *et al.*, 2007) and human leukotriene C_4 synthase (LTC_4S), represent different hydrophobicities and detergents necessary for reconstitution. The carboxylase is predicted to have a large hydrophilic domain and is solublized in CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), while LTC_4S is mainly hydrophobic and purified in Triton X-100. The detergents are subsequently removed by dialysis and substituted by dimyristoyl phosphatidylcholine (DMPC) to form 2D crystals under controlled conditions. Parameters such as temperature, salt, glycerol, length of dialysis, lipid-to-protein ratio (LPR) and detergent concentration have been tested. Now identical buffer conditions with and without protein were used to examine the influence of the presence of proteins on the membrane size and morphology and whether

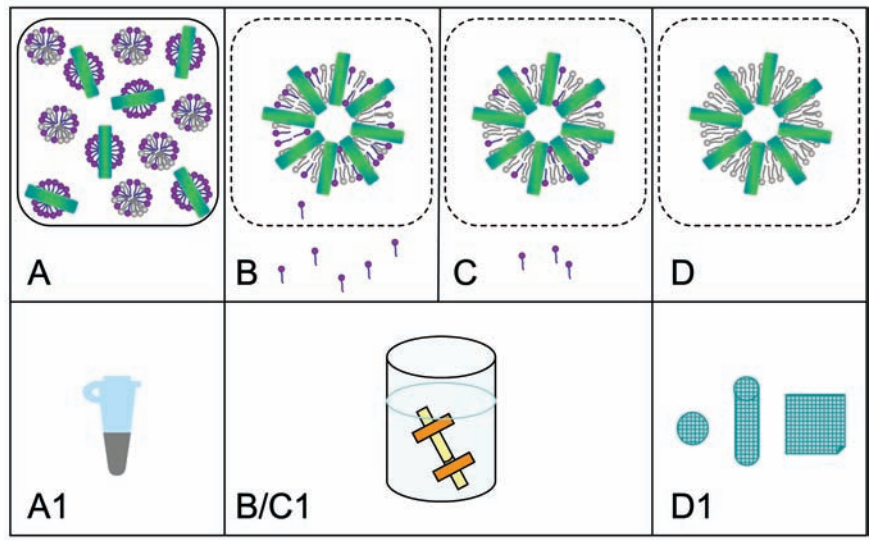

Figure 1. Schematic representation of 2D crystallization of membrane proteins by dialysis. In Figures A and A1 the purified and detergent-solubilized membrane protein is mixed with lipid-detergent micelles. The protein is depicted in green, the detergent in purple, and the lipid in grey. Figures B, C and B/C1 illustrate the detergent removal by dialysis against detergent-free buffer. After dialysis, the protein is reconstituted (D). Under the optimal conditions, 2D crystals form. These usually have a distinctive membrane morphology such as vesicles, planar-tubular, or sheet-like (D1, left to right). Mixtures of the morphologies are also frequently observed.

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these lipid-detergent dialysis tests can be used as controls.

Screening for 2D crystals is performed on negatively stained grids, as these provide a rapid means of preparing a large number of samples (Kühlbrandt, 1992). While the negatively stained specimens have good contrast, they only provide lower resolution information. This is in most cases not beyond 15Å resolution, yet is more than sufficient to identify the two main criteria for success of 2D crystallization experiments: (1) crystal order and (2) size. While electron cryo-microscopy and image processing are essential for evaluating the achievable resolution of the crystals (Fujiyoshi, 1998), negative stain is the quickest means of optimizing crystals for order and size as well as possibly a reduction in mosaicity. Initially it is established that a satisfactory number of membranes are present on most grid squares, which will be necessary to allow for proper assessment of the sample based on an adequate number of proteoliposomes. The first successful step towards obtaining 2D crystals is in fact reconstitution of the membrane protein. Freeze-fracture is a very valuable tool in not only determining reconstitution, but also in giving a good indication of the relative amount of protein compared to lipid under the given conditions. Next, an overall impression of the membrane morphology is noted, which can be subdivided into vesicles, planar-tubular membranes or sheets (Fig. 2a – c). The specimens can be composed of either a single morphology or a mixture. Both types of samples will contain a range of membrane sizes. The most important point to note about the membrane dimension is that a sufficient number of membranes of a certain size is available for imaging.

Large numbers of membranes need to be inspected for crystallinity either with the help of a CCD camera and Fourier transform or on film, allowing for easier assessment of large crystals for size and mosaicity, as initial steps of 2D crystallization at suboptimal LPRs can result in less than 10% of proteoliposomes containing crystals (Zhao, Kanaoka, Irikura, Lam, Austen and Schmidt-Krey, in preparation). A similar observation was also made for vitamin K-dependent γ-glutamyl carboxylase (Schmidt-Krey *et al.*,2007), where the first crystals were few and limited in location to 1 or 2 grid squares. This was not caused by uneven grid preparation as the grids were otherwise uniform in appearance (not shown), but can be attributed to a low number of crystals.

In addition to testing the standard protein preparations for different crystallization parameters, it was found that dialysis of lipid-detergent mixtures under identical conditions, yet without protein, proved to be a valuable tool and control for assessing buffer conditions for at least two eukaryotic membrane proteins. This can be particularly helpful if only small amounts of protein are available, as well as for assessing buffers for their value in membrane formation. To test the feasibility of obtaining useful data from lipid-only dialysis experiments, buffers and lipid-detergent mixtures were used that reproducibly resulted in well-ordered 2D crystals in the presence of protein, namely human vitamin K-dependent γ-glutamyl carboxylase (Schmidt-Krey *et al.*, 2007) and human leukotriene C_4 synthase (LTC_4S) dialysis buffers (Zhao, Kanaoka, Irikura, Lam, Austen and Schmidt-Krey, in preparation).

Human carboxylase forms the largest continuous ordered arrays when it is induced to form planar-tubular crystals (Fig. 2b), while the most well-ordered LTC_4S 2D crystals are sheets that are on average at least 2μm in size (Fig. 2c). Both proteins crystallize in different morphologies and symmetries under controlled conditions, with the largest number of crystal forms observed for LTC_4S, namely, p2, p3, p312, and two different unit cell sizes of p321. In both cases it was found that the protein crystallizes before complete detergent removal, yet, as expected, the crystals proved far more stable, usually for at least several months, after elongated dialysis time. This stability could be extended much further by storing the 2D crystals at -80°C with the added advantage to prevent occurrence of effects observed in regular storage such as stacking (Schmidt-Krey, 2007).

By testing a lipid-detergent mixture without protein dialysed against detergent free buffer, liposomes in the micron-range of ~2-4μm and over 10μm in size could be obtained with characteristics typical of a particular buffer used. In the case of a DMPC-detergent mixture dialysis against vitamin K-dependent γ-glutamyl carboxylase buffer (25 mM Tris-HCl at pH 7.2, 20% glycerol, 250 mM NaCl, 10 mM β-mercaptoethanol) this resulted in planar-tubular liposomes (Fig. 3a and b) under similar conditions as the 2D crystallization conditions, as had also previously been observed for both the 2D crystals as well as DMPC planar-tubular membranes grown at 20°C. With the LTC_4S buffer (50 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM 2-mercaptoethanol, 20% glycerol, 10 mM GSH, and 50 mM KCl), similar membrane sizes, morphologies and breakage could be observed as for the 2D crystals of the protein (Fig. 3c and d). The main differences with the carboxylase buffer appear to be the cleaner background in the lipid/detergent-only dialysis as well as the larger membrane size. The difference in membrane size can possibly be explained by additional curvature of the planar-tubular carboxylase crystals imposed by the protein

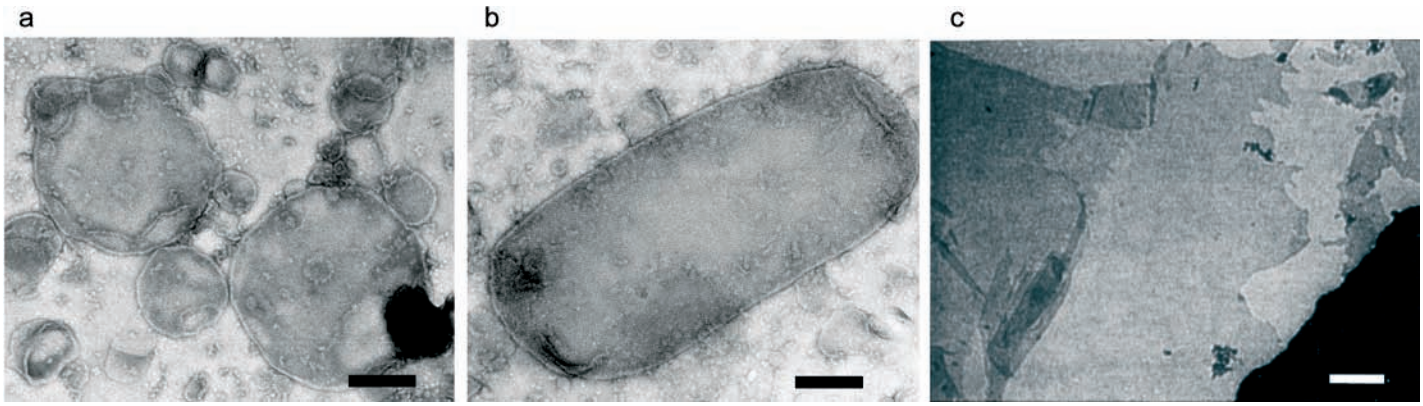

Figure 2. Images of typical two-dimensional crystals used for structural studies of (a) and (b) human vitamin K-dependent γ-glutamyl carboxylase and (c) human leukotriene C_4 synthase with (a) vesicle, (b) planar-tubular and (c) sheet morphology, respectively. The scale bars are (a) and (b) 200nm and (c) 1μm, respectively.

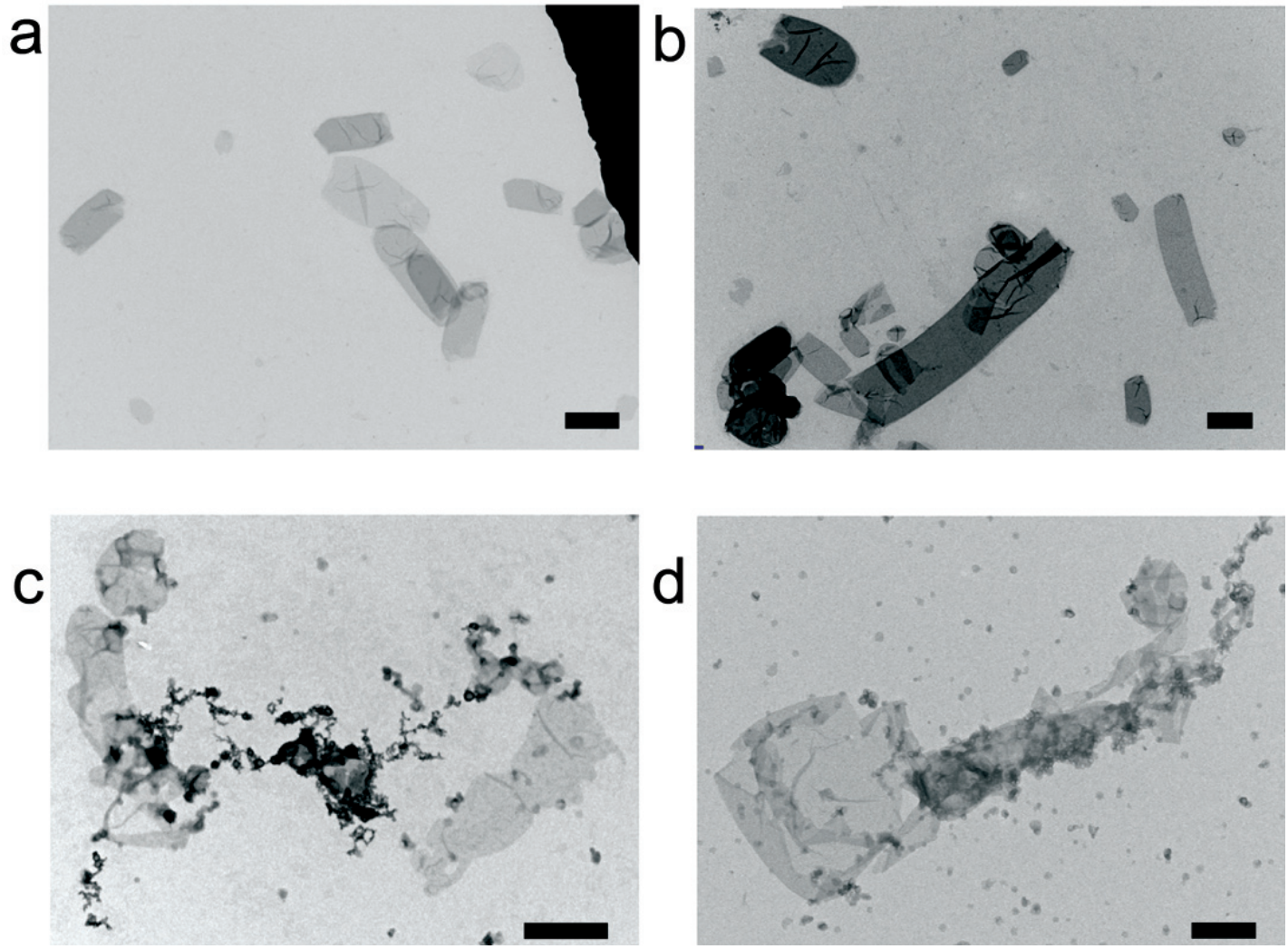

Figure 3. Images of a lipid-detergent mixture without protein after dialysis. (a) and (b) show planar-tubular membranes of DMPC-CHAPS dialysed against the carboxylase buffer. (c) and (d) are micrographs of a DMPC-Triton X-100 mixture dialysed against LTC_4S buffer. The scale bars are 1μm.

geometry, which is highly packed within the 2D crystals.

It was found that dialysis of detergent-solubilized lipid is a valuable indicator of membrane formation when optimizing buffer and lipid conditions. These tests without protein present could be particularly useful in cases where protein is difficult to produce or obtain. Consequently preliminary testing with the buffer can be done on lipid-detergent mixtures as well as used for controls of membrane formation under a given buffer condition. Similar tests will now have to be undertaken on other crystallizable membrane proteins to observe if this trend is general or can only be used in the screening of the LTC_4S and carboxylase crystallizations. ■

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