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Two-dimensional crystallization conditions of human leukotriene C₄ synthase requiring adjustment of a particularly large combination of specific parameters

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Abstract

Human leukotriene C₄ synthase (LTC₄S) forms highly ordered two-dimensional (2D) crystals under specific reconstitution conditions.

It was found that control of a larger number of parameters than is usually observed for 2D crystallization of membrane proteins was necessary to induce crystal formation of LTC₄S. Here we describe the parameters that were optimized to yield large and well-ordered 2D crystals of LTC₄S.

Careful fractioning of eluates during the protein purification was essential for obtaining crystals. While the lipid-to-protein ratio was critical in obtaining order, four parameters were decisive in inducing growth of crystals that were up to several microns in size. To obtain a favorable diameter, salt, temperature, glycerol, and initial detergent concentration had to be controlled with great care. Interestingly, several crystal forms could be grown, namely the plane group symmetries of p2, p3, p312, and two different unit cell sizes of plane group symmetry p321.

Keywords

Two-dimensional crystallization; membrane protein; eukaryotic; structure; electron crystallography; cryo-EM

Eukaryotic membrane proteins constitute the majority of medically significant drug targets. Recent years have seen an increase in membrane protein structures with major contributions from X-ray crystallography (Stroud *et al.*, 2009), yet progress is far from comparable to structural studies of soluble proteins, and the number of eukaryotic membrane protein structures is even smaller (Werten *et al.*, 2002).

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While two structures of leukotriene C₄ synthase (LTC₄S) have been obtained from 3D crystals (Ago *et al.*; Molina *et al.*, 2007), questions fundamental to the understanding of the structure-function relationship of LTC₄S still remain unanswered. Specifically, information on whether the substrate leukotriene A₄ approaches the active site from within or above the bilayer as well as the reaction mechanism is needed. One important approach complementary to X-ray crystallography in gaining insight into these questions is two-dimensional (2D) crystallization and electron crystallography, which allow for functional studies within close-to-native conditions of a phospholipid bilayer. These experiments not only make it possible to study the protein itself, but can also contribute information critical for crystallizing other membrane proteins. Several laboratories are in the process of undertaking thorough investigations of 2D crystallization conditions for membrane proteins (Jap *et al.*, 1992; Stahlberg *et al.*, 2001; Mosser, 2001; Kühlbrandt, 1992, 2003; Schmidt-Krey, 2007; Signorell *et al.*, 2007; Vink *et al.*, 2007). Recently, key trends derived from a large number of 2D crystallization experiments were summarized and resulted in important conclusions such as the high frequency of successful 2D crystallizations with dimyristoyl phosphatidylcholine (Kühlbrandt, 2003; Schmidt-Krey, 2007). An even more thorough understanding of 2D crystallization, necessitating data from successful crystallization trials combined with methods development (Cheng *et al.*, 2007; Stahlberg *et al.*, 2001; Vink *et al.*, 2007), will likely make it feasible to study much larger numbers of membrane proteins at an increased rate in the not too distant future. This will allow for the focus and effort in electron crystallography to be on investigating structure-function relationships.

LTC₄S is a member of a protein superfamily (Jakobsson *et al.*, 1999), of which the 2D crystallization conditions of microsomal glutathione transferase 1 (MGST1) were examined in relatively great detail (Schmidt-Krey *et al.*, 1998). With the aim of growing 2D crystals as well as contributing to the understanding of 2D crystallization and its critical parameters, a thorough investigation of crystallization conditions was also conducted for LTC₄S. While a large range of parameters was tested for MGST1 once crystals were obtained, LTC₄S required numerous conditions to be tested before successful crystallization, likely due to the large number of parameters that needed coordination.

Materials and Methods

Over-expression and purification

LTC₄S was over-expressed and purified (~1mg/ml) according to Schmidt-Krey *et al.* (2004). Protein fractions were either pooled or eluted separately from S-hexylglutathione agarose.

2D crystallization trials

Purified protein, solubilized in 1% Triton X-100 (TX-100) and 0.5% Na-deoxycholate (Na-DOC) was mixed with phospholipids and dialyzed against detergent-free buffer (50mM HEPES, pH 7.6, 1mM EDTA, 10mM β-mercaptoethanol, 20% glycerol, 10mM GSH, 10–100mM KCl) for 8–21 days at 20–24°C. Dialysis tubing with a 10,000 MWCO (1cm flat width) was used. Phospholipids tested were dimyristoyl phosphatidylcholine (DMPC) and dioleoyl phosphatidylcholine (DOPC). Protein-detergent-lipid mixtures (75–100μl) were dialysed against 250ml of detergent-free buffer. Samples were transferred to 1.5ml Eppendorf tubes and stored at room temperature or plunge frozen in 3–5μl aliquots for later use.

Freeze-fracture

A droplet of sample, placed between copper plates, was plunge-frozen in liquid nitrogen-cooled ethane. Freeze-fracturing was performed in a Balzers BAF 400T at 3×10^{-7} mbar keeping the specimen stage at –130°C. Shadowing with platinum/carbon was at an angle of 45° and with pure carbon at 90° reinforcing the heavy metal replica. Thawed replicas were cleaned from

organic material in chromosulfuric acid, washed in water, placed on Formvar-coated copper grids and viewed with an EM208S electron microscope (FEI) equipped with a 1Kx1K slow-scan CCD camera (Tietz, TVIPS GmbH, Munich, Germany).

Electron microscopy

After dialysis, volumes of 2–4 μ l were negatively stained with 1% uranyl acetate. Membranes were evaluated at 120kV in a Pentaholder (JEOL) with a JEM-1400 (equipped with Gatan Orius SC1000 and Ultrascan 1000 CCD cameras) or with a CM120 electron microscope (EM). The crystals were prepared for cryo-EM by the back-injection method (Wang and Kühlbrandt, 1991) with 4.0–4.5% trehalose. Cryo-EM data was collected with a JEOL 3000SFF and an FEI Tecnai G2 Polara at 300kV and 4K on SO-163 film under either flood-beam or spot-scan (exposure time: 45ms/spot) conditions (Downing 1991) at nominal magnifications ranging from 50,000–70,000 (Fujiyoshi *et al.*, 1998). Images of well-ordered crystals were obtained. Electron diffraction data collection was not as successful as imaging and needs to be tested further.

Image processing

Micrographs were evaluated by optical diffraction, and a Zeiss SCAI scanner (7 μ m step size) was used to scan the best image areas of 4Kx4K pixels. Images were processed with the MRC programs (Crowther *et al.*, 1996).

Results and discussion

Elution and lipid-to-protein ratio

Careful selection of particular fractions eluted from S-hexylglutathione agarose was the main factor in the successful 2D crystallization of LTC₄S in combination with the lipid-to-protein ratio (LPR) (Fig. 1).

While initial purification batches were pooled and then tested for 2D crystallization, these only occasionally resulted in crystals. A high degree of success was ensured by eluting 2–5 fractions and separately testing these for crystallization. Furthermore, multiple LPRs were evaluated for each fraction to exclude failures to crystallize due to co-purified lipid and thus a change in the required LPR. Fractions were concentrated, if necessary, to ≥ 0.3 mg/ml and up to several milligrams in concentration, which did not appear to have an effect on crystallization. Usually ~50–80% of eluates would readily form ordered arrays.

Not surprisingly, the LPR played an equally critical role in crystallization, as has been observed for other membrane proteins (Schmidt-Krey, 2007; Kühlbrandt, 2003). Freeze-fracture images were used on the first crystallization trials to determine starting points for selecting LPRs (not shown), and subsequent crystallization experiments were examined in negative stain. Table I illustrates the dramatic improvement in percentages of crystals with decreasing LPRs. While the molar LPRs are unusually small, this is very likely due to co-purified lipid in protein preparations, which was later found to necessitate adjustment of LPRs between purification batches. What this shows, rather than the definitive LPR required for crystallization, is the absolutely minimal changes in LPR necessary to induce an effect. This first occurs in experiment B, where the gradual decrease in LPR by steps of 0.1 results in improving percentages of crystals between 2.5–100% for a range of LPRs between 0.3–0.6. In C, LPRs of 0.10, 0.15, 0.20, and 0.25 (only LPR=0.20 shown in Table I) resulted in identical percentages of crystals (100%) and degrees of mosaicity. In experiment D, however, an absolutely minimal LPR change between 0.10 and 0.05 resulted in a nearly entire elimination of mosaicity. These experiments also highlight several important conclusions that are generally applicable to 2D crystallization trials. Firstly, in experiment A, only 2% of membranes selected contained

crystalline arrays. If the grids had not been screened thoroughly, the first crystals might not have been identified, with detection leading to rapid improvements. Secondly, from obtaining first small crystals in 2% of images to reproducibly growing large single crystals took only three additional experiments. As only the LPR was varied, this emphasizes how critical this factor is. Lastly, each one of these experimental steps (A–D) was constituted of just four different LPRs, demonstrating that once first ordered arrays are observed, crystal improvements through LPR adjustments can be achieved with relatively few experiments and steps, which require careful and thorough screening.

DMPC and DOPC were used for crystallization trials and resulted in equally large membranes under the right buffer and temperature conditions. Other lipids were not tested as DMPC and DOPC showed promise in initial experiments.

Salt concentration

Salt is believed to affect interactions of hydrophilic domains (Kühlbrandt, 1992) and thus influence crystal size. The KCl concentration was found to have a decisive impact on membrane dimensions. Concentrations of 100mM KCl stopped proteoliposomes growth at ~100–200nm (not shown), and limited the morphology to nearly exclusively vesicles. In fact, the KCl concentration-dependence on membrane size was so critical that even minimal variations from the ideal 50mM restricted the membrane size significantly. This fine balance might be required due to higher salt concentrations causing excessive interactions of hydrophilic domains and thus increased aggregation preventing growth. The difference in morphology could be explained by larger vesicles breaking into sheets upon grid preparation as seen in some sheets (Fig. 1).

Detergents and glycerol concentration

Nearly identical observations were made regarding the detergent concentration before dialysis as had been made for the concentration dependence of detergent for the 2D crystallization of MGST1 (Schmidt-Krey *et al.*, 1998), where a slower rate of dialysis lead to an increased crystal size. An unusually high concentration of 1% TX-100, in combination with carefully adjusted KCl, reproducibly resulted in many large sheets with a diameter of frequently several microns. These could measure up to 10–20 μ m in size.

The lipid was solubilized in Na-DOC as this detergent had been used successfully in the 2D crystallization of other membrane proteins (Schmidt-Krey *et al.*, 1998; 2000). Additional detergents were not tested due to the positive outcome with Na-DOC. While Na-DOC can act denaturing on membrane proteins (Kühlbrandt, 1988), it is removed quickly by dialysis, and it is likely that Na-DOC is replaced promptly by TX-100. As the TX-100 concentration is exceptionally high, it likely protects LTC₄S from excessive contact with Na-DOC. The dialysis time was varied between 8–21 days with protein batch and detergent concentration.

Similar to MGST1, 20% glycerol was found to be an important factor in obtaining the largest possible crystals.

Temperature

The dialysis temperature played a significant role in obtaining large crystals. 23–24°C was optimal for obtaining large crystals in the micron-range. Temperatures $\leq 22.5^{\circ}\text{C}$ produced crystals $< 1\mu\text{m}$ and a large number of considerably smaller aggregated crystals (Fig. 2). While vitamin K-dependent γ -glutamyl carboxylase forms large 2D crystals under the DMPC phase transition temperature (T_c) (Schmidt-Krey *et al.*, 2007), a similar temperature-dependence of crystal size as for LTC₄S was observed with MGST1 (Schmidt-Krey *et al.*, 1998). Trials of LTC₄S with DOPC ($T_c = -20^{\circ}\text{C}$) resulted in similarly-sized membranes as with DMPC under

identical conditions. This leads to the conclusion of a temperature-dependence for LTC₄S membrane size rather than a specific lipid-dependence with its associated T_c .

Circumventing stacking of 2D crystals

Some LTC₄S purification batches resulted in numerous stacked crystals (not shown). Stacking usually occurred within the first week of removing samples from dialysis. Several approaches were taken to overcome this problem. First, cryo data was collected of crystals immediately following dialysis. As the crystallization was highly reproducible, pre-screening by negative stain could be omitted. Crystallizations could not be repeated frequently since protein amounts were quite limited at the time, and it was thus not feasible to set up enough crystallization trials. A second alternative was freezing of a large number of grids after the crystallization had been newly sampled. This approach, however, prevented further adjustment of freezing conditions, should a batch of cryo grids have required modification. A speculation was that selective data collection of single-layered crystals would not be possible at higher tilt angles as the number of crystal layers would be obscured. Tests at 45° tilt worked unexpectedly well in the selection of single crystals. The best means by far of avoiding stacking was to plunge-freeze small aliquots of 5–10 µl into liquid nitrogen immediately after removal from dialysis, to store these at –80°C, and then thaw them for cryo grid preparation (Schmidt-Krey, 2007). There are first indications that stacking of the crystals is concentration-dependent. Another cause for stacking might very well be helix 5, which protrudes from the membrane (Ago *et al.*, Molina *et al.*, 2007).

Symmetries

LTC₄S has the potential to form several different crystal plane group symmetries. The first and to date most commonly observed crystal form is p321 with unit cell dimensions of $a=b=73.4\text{\AA}$, $\gamma=120^\circ$ (Fig. 3a). Subsequently, p2, p3, p312, and a second p321 type were identified. All projection maps agree well with the first projection structure (Schmidt-Krey *et al.*, 2004) and the structures determined by X-ray crystallography: four transmembrane α -helices can be identified yet helix 5 cannot be visualized in projection as expected. The first distinguishing feature of the second p321 crystal is the larger unit cell size of $a=b=83.0\text{\AA}$, $\gamma=120^\circ$ (Fig. 3b). Upon closer examination of the unit cells, a marked rotation of both of the trimers in the larger unit cell with respect to the crystals in the smaller unit cell becomes evident. In Figure 3, this is indicated by the small circle signifying the center of the unit cells and the orange outlines of two each of the outer α -helices forming the crystal contacts between two oppositely oriented trimers. While the placement of these pairs of α -helices in respect to the neighboring trimer is nearly parallel in the smaller p321 crystals, in the larger p321 crystals the outer pairs of α -helices have been rotated away from each other and the center of the unit cell, keeping only one contact between one each of the α -helices of the two trimers. The large number of crystal forms and different possible crystal packings suggest a sizeable quantity of possible crystal contacts.

By far the largest number of crystals was of p321 symmetry with the smaller unit cell size, possibly indicating that this is the tightest crystal contact. These crystals were also of the highest quality (Schmidt-Krey *et al.*, 2004). The other crystal types were found more recently and need further investigation of both the reasons for their formation as well as possible quality. It is plausible that the LPR plays a role in inducing the plane group symmetries. An influence of the symmetry on crystal flatness will be investigated as optimal flatness is critical in collection of tilted data.

Conclusions

An unusually large number of parameters was found to be important in obtaining big and well-ordered 2D crystals of LTC₄S. Order could be induced by a combination of (1) dividing/ selecting fractions during the purification as well as (2) a low LPR. The size required coordination of four factors: salt, temperature, glycerol, and initial detergent concentration. First evidence of the possibility of different numbers of subunits (unpublished data) might suggest a cause for this extraordinarily large number of crystallization conditions.

Efforts towards automation of screening (Cheng *et al.*, 2007), crystallization equipment (Engel *et al.*, 1988; Stahlberg *et al.*, 2001) and image processing (Gipson *et al.*, 2008) will be of tremendous benefit not only towards further development of the method, but also towards increasing the understanding of membrane protein structure. The results of the crystallization studies of LTC₄S will be used to further understand the reaction mechanism as well as contribute to 2D crystallization trials of other eukaryotic membrane proteins.

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Figure 1.

Images of typical 2D crystals of LTC₄S. Crystals are continuous, without mosaicity, and display sheet-like morphologies in most instances. Few vesicles are present. (a) The lattice extends continuously to the uneven edge of the crystal. Optical diffraction (inset) shows that the image area contains a single crystal. (1c–d) are parts of the same membrane and illustrate the fractures that can occur upon contact with the carbon support film. As the fractures and uneven membrane edges arise during grid preparation, the lattice is continuous even in the uneven edges. Due to the low magnification the lattice in the overview images (c–d) cannot be discerned. Scale bars are (a) 50nm and (b–d) 1 μ m.

Figure 2. Temperatures $\leq 22.5^{\circ}\text{C}$ resulted in (a) crystals (arrows) $<1\mu\text{m}$ in size and (b) a large number of considerably smaller aggregated crystals shown by the arrow and barely visible at the edges of the black aggregates. These are significantly smaller than the crystal sheets of several microns shown in Figure 1.

Figure 3.

Projection maps of crystals with p321 plane group symmetry and unit cell dimensions of (a) $a=b=73.4\text{Å}$, $\gamma=120^\circ$; (b) $a=b=83.0\text{Å}$, $\gamma=120^\circ$. The maps are truncated at 7.5Å resolution to visualize the transmembrane α -helices. Centers of unit cells are indicated by circles and the outer α -helices forming the crystal contacts between trimers are boxed.

Table 1

The percentage increase of 2D crystals by minimal adjustments of lipid-to-protein ratios

LPR [‡]	images collected	# images of crystals [*]	percentage	mosaicity	experiment
0.875	188	4	2.0	+	A
0.6	40	1	2.5	+	B
0.5	50	14	28.0	+	"
0.4	50	17	34.0	+	"
0.3	50	50	100.0	+	"
0.2	50	50	100.0	+	C/D
0.1	50	50	100.0	+	D
0.05	50	50	100.0	mostly single^{**}	"
0.025	50	50	100.0	+	"
0	50	0	0	N/A	"

[‡]The LPR is in terms of molar ratios. The low LPR values are likely due to co-purified lipids.

^{*}Determined by optical diffraction.

^{**}Continuous crystals without mosaicity.