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REVIEW

Protein crystallization

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Photosynthesis is one of the most important chemical reactions in the world. In green plants, the light reactions of oxygenic photosynthesis are fulfilled by a series of protein complexes in the thylakoid membrane. Photo-energy absorption, transfer, and conversion are accomplished by the interplay of the light-harvesting system and the reaction center. Three-dimensional structural information for these protein complexes provides the basis for understanding the mechanism of photosynthesis. However, most of the proteins that participate in the light reactions of photosynthesis are membrane proteins, and structure determination of membrane proteins is much more difficult than that of soluble proteins because of their extremely hydrophobic character.

The main methods for solving the structure of biomacromolecules are X-ray crystallography, nuclear magnetic resonance (NMR), and electron microscopy (EM). X-ray crystallography is currently the most powerful method for structure determination of biomacromolecules. There is now a total of 53,917 macromolecule structures stored in the Protein Data Bank (PDB), most of which (48,618; about 85%) has been solved using X-ray crystallography. Less than 1% of these crystal structures are of membrane proteins, and there are less than 100 structures of photosynthesis-related proteins (31.3.2009 data) (http://www.rcsb.org/pdb/home/home.do, http://www.mpdb.ul.ie/).

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The basic procedures for X-ray crystallography are: protein sample preparation, crystallization, diffraction data collection, phase determination, electron density analysis, and model building. The most important, and also the most difficult steps in X-ray crystallography are obtaining a good single crystal and determining phase information. Despite technical and methodological advances, getting well-diffracted single crystals is also a rate-limiting step, and still remains a major obstacle in X-ray crystallography.

Principles of protein crystallization

The process of crystallization is ordering the arrangement of protein molecules. Crystals are only formed when protein molecules are in the supersaturated state and aggregate in an ordered fashion. When a protein solution and crystallizing reagents are mixed, protein concentration is either supersaturated (crystallization can occur) or unsaturated (the protein is dissolved in the liquid phase and crystals cannot form), as shown in Fig. 1 (Weber 1997). The supersaturated region can be divided further into three regions: the metastable region (low supersaturation), the labile region (moderate supersaturation), and the precipitate region (high supersaturation where precipitation of amorphous protein occurs).

The crystallization process can be divided into two steps: nucleation where crystalline nuclei are formed by ordered aggregation of large numbers of protein molecules and crystal growth, i.e., numerous protein molecules aggregate with these nuclei in an ordered fashion, making them reach a critical size. The nucleation step is only possible when the concentration of the protein solution is in the labile region, while the second step can occur in both the metastable and labile regions. Thus, the prerequisite for

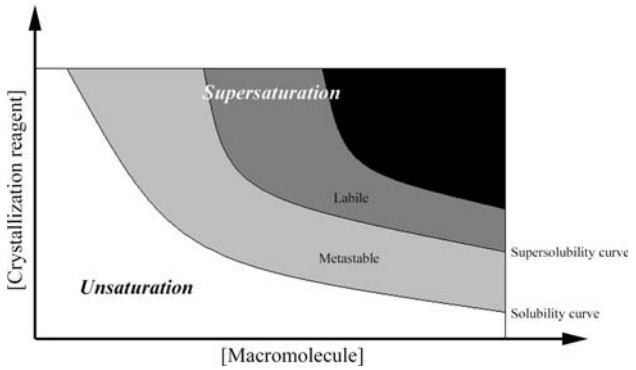

Fig. 1 Phase diagram. Different phases are shown in different shades of gray. Saturation level increases as the color goes from white to black. The *white region* represents unsaturated solutions where existing crystals will dissolve. The unsaturated region is divided from the supersaturated region (shown in *different shades of gray*) by the solubility curve. Supersaturated solutions can be divided further into three regions. The metastable phase where only crystal growth will occur, is shown in *light gray*. The labile phase where nucleation and growth compete, is shown in *dark gray*. These two regions are divided by the supersolubility curve. The precipitate region (shown in *black*) has the highest supersaturation and precipitation of amorphous protein will occur in this region

obtaining crystals is to maintain the protein concentration in the metastable and/or labile regions.

In an ideal crystallization process, protein concentration will be in the labile region and nuclei will be formed. After a few nuclei separate out, the protein concentration, although still supersaturated, drops into the metastable region. As numerous protein molecules separate out and aggregate with the existing nuclei, no more crystal nuclei are formed, so the number of nuclei will be limited and large crystals can be obtained. In this process, the main point is to keep the protein concentration at a low supersaturation, in other words, the protein concentration should be near the supersolubility curve of the labile region (Fig. 1).

Methods and techniques for protein crystallization

The success of crystallization depends on finding appropriate reagents and conditions under which protein molecules are arranged in an ordered fashion and protein concentration is held in the supersaturated state, so that the crystals can separate out. Crystallization reagents consist of precipitants, buffers, and sometimes additives. *Precipitants* can be of all kinds such as PEGs, salts, and other chemicals used at high concentrations, and their effect is to push proteins into the supersaturated phase. *Buffers* maintain pH stability in the crystallization system and are used at low concentrations (usually 20–200 mM). *Additives* can be of all kinds chemicals, such as polymers, organics, salts, reducing agents, and carbohydrates, or amino acids. The amount of additives in the crystallizing system is very low, as they do not affect the saturation state of protein solutions, but rather manipulate sample–sample and sample–solvent interactions, and consequently facilitate the ordered arrangement of protein molecules. There are many reporting that using additives can improve the quality of macromolecular crystals (Liu et al. 2004; Huet et al. 2008).

Techniques for crystallization include the vapor diffusion, batch crystallization, and dialysis methods (Luft and DeTitta 1997). Vapor diffusion is the most popular method and includes the hanging-drop and sitting-drop vapor diffusion techniques (Chayen and Saridakis 2008). In this technique, a droplet (usually 1–10 μl) and a reservoir with a larger volume (usually greater than 100 μl) are sealed within a closed system. The droplet containing the protein solution and crystallization reagents equilibrates by vapor diffusion with the reservoir solution which has a higher precipitant concentration than that of the protein-containing drop. The protein concentration increases to supersaturation through reduction in the solution volume of the protein-containing drop.

In the batch crystallization method, the protein solution is mixed directly with the crystallizing reagent which brings the protein solution into a supersaturated state, and crystallization occurs after incubation. The volume of the drop in the conventional batch method is quite large, but in a newly developed method, namely the micro-batch crystallization method, the drop is sealed using oil, and its volume can be less than 1 μl (Chayen 1998). The crystals of chlorophyll binding protein 43 (CP43) of photosystem II (PSII) from spinach were obtained using the micro-batch method under oil (Kern et al. 2005). Although, the diffraction of the crystals obtained in this case was not ideal, useable crystals were obtained, in contrast to the vapor diffusion and dialysis methods which did not yield any crystals.

In the dialysis method, the protein solution is also mixed directly with the crystallizing reagent. Here, however, the protein concentration reaches supersaturation gradually by allowing the mixed drop to equilibrate with a more highly concentrated solution through a semipermeable membrane. Photosystem I from cyanobacteria was crystallized using the dialysis method and the crystal diffracted to 4 Å (Witt et al. 1992; Fromme and Witt 1998).

Screening and optimization processes for membrane protein crystallization

Membrane proteins are only stable in solution, when appropriate detergents are added because of their extremely hydrophobic character. Choice of detergent is, therefore,

very important in membrane protein crystallization, which is otherwise similar to soluble protein crystallization, both in terms of principles and techniques.

The initial crystallization screen of membrane proteins is usually conducted using commercial crystallization kits, just as for soluble proteins. Many commercial crystallization kits specialized for membrane protein crystallization are available. Alternatively, Iwata summarized crystallization conditions that have been used successfully for crystallizing some all-alpha membrane proteins, and designed a screening kit for all-alpha membrane proteins. These recipes have been published and are simple to follow (Iwata 2003).

Finding an initial crystallization condition for membrane proteins to yield small crystals may not be very difficult, but further optimization to generate crystals that are suitable for X-ray crystallography is a challenging task. In most cases, the initial crystallization conditions which yield small crystals are not the optimal conditions for crystallization, and hence we need to adjust various parameters in order to obtain the best quality crystals. During the initial steps of optimization, various parameters, such as protein concentration, the kind and concentration of the precipitant, the pH value, the kind and concentration of detergent, additives, and temperature require some adjustment to find the optimal conditions.

However, all other considerations are secondary to the protein purity and homogeneity which are the prerequisites for successful protein crystallization. In most cases, impurity or heterogeneity will hinder the ordered packing of the target protein molecules, and thus influence the quality of the crystals. One example of this is the crystallization of PSI from cyanobacterium. Crystals of trimeric PSI were first obtained using the dialysis method, and diffracted at 6 Å resolution. Experiments showed that the presence of one monomer per 10,000 trimeric molecules prevents the growth of large well-ordered single crystals of trimeric PSI. Further improvements to methods for the isolation of trimeric PSI from monomeric PSI, as well as the optimization of the crystallization conditions, led to better crystals that diffracted at 4 Å resolution (Fromme and Witt 1998; Fromme 2003). In addition to protein quality, the nature of detergents and additives is very important for membrane protein crystallization and is discussed more extensively below.

Detergents

The membrane proteins have a large hydrophobic surface area, and hence an appropriate detergent is necessary for the purpose of the membrane protein solubilization in the expression, purification, and crystallization procedures. Once the membrane proteins have been stabilized with detergent, they can be crystallized using suitable reagents and conditions. A given detergent may be suitable for solubilizing and stabilizing a specific membrane protein, but may hinder the protein molecule interactions that are necessary for crystallization to occur. If this is the case, changing or adding other kinds of detergent is necessary for the purposes of crystallization. Mixed detergents are required in some cases when the desired results cannot be obtained with a single kind of detergent. For example, well formed 3D crystals of the oxygen-evolving photosystem II reaction centers (RCII) from spinach and pea could only be obtained by utilizing detergent mixtures (Adir 1999). Detergents which have been widely used with success in membrane protein crystallization, at concentrations slightly higher than their CMC (critical micelle concentration), include *N*-Octyl-β-D-Glucopyranoside (β-OG), *N,N*-Dimethyldodecylamine-*N*-Oxide (LDAO), *N*-Dodecyl-β-D-Maltopyranoside (β-DDM), *N*-Decyl-β-D-Maltopyranoside (β-DM), and Octyltetraoxyethylene (C8E4) (http://www.mpdb.ul.ie/pickstat.asp).

Additives

The effect of additives cannot be ignored, even though their concentration is very low. In some cases, if particular additives are not used, crystals, or well-diffracting crystals, cannot be obtained. In addition to conventional additives, some other additives such as amphiphiles and lipids are important in membrane protein crystallization. Amphiphiles are a group of small molecules, which have one polar end and one hydrophobic end. Amphiphiles do not form micelles on their own, but together with detergent they can form mixed micelles (Timmins et al. 1991). Membrane proteins, with such mixed micelles bound on their surface, may have a larger hydrophilic region and more space for molecular contacts. This consequently promotes the order of crystal packing. Heptane-1,2,3-triol and benzamidine hydrochloride are the two additives, which have been used most successfully so far. Crystals of the photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis* (Deisenhofer and Michel 1989) and of the reaction center from the nonsulfur purple bacterium *Rhodobacter sphaeroides* (Allen 1994) were successfully improved when these two additives were used. Lipids are another very important additive that aids membrane protein crystallization. Lipids may interact with membrane proteins and help to improve their conformational homogeneity. In some cases, large scale purification using chromatography can be employed to yield pure protein, but this treatment may cause protein de-lipidation and result in failure of crystallization. Under such circumstances, adding lipid is crucial for obtaining crystals. Cyanobacterial cytochrome b6f (Zhang et al. 2003) and

LHC-II from spinach (Liu et al. 2004) cannot be crystallized without adding a specific lipid.

Apart from simply altering crystallization conditions, other techniques can be used to optimize crystal quality. Seeding is one technique that has been used widely and successfully. Seeding techniques can be classified into microseeding and macroseeding, according to the differences in the methods for producing nuclei. In microseeding, crystal "seeds" are produced by crushing unusable crystals into nuclei, while in macroseeding a crystal "seed" is produced by etching the surface of a small single crystal (i.e., by soaking the crystal briefly in an undersaturated solution to slightly melt the crystal and generate a fresh surface.) The seed produced is then transferred into a growth solution, which keeps the protein concentration in the metastable phase, so that the nucleus will develop into large crystals without formation of further nuclei (Bergfors 2003). Seeding techniques were successfully used for optimizing the crystals of PS-I from the cyanobacterium *Synechococcus elongates* (Fromme and Witt 1998).

In some cases, particular proteins cannot be crystallized alone because they have a large content of flexible loops or inhomogeneous conformation. Soaking or co-crystallizing these proteins with their substrates or product analog, inhibitors, or ligands can be used to optimize the shape and/or quality of resulting crystals as these molecules may stabilize protein conformation. These methods have been widely reported (Heras and Martin 2005) and will not be discussed further.

In addition, some optimization techniques focusing specifically on membrane protein crystallization have been developed. Here, we will briefly introduce two techniques which have been used successfully for optimization of membrane protein crystals. First Lipid Cubic Phase (LCP) is a method based on the fact that some phospholipids (usually monoolein) can form a cubic phase at suitable temperatures and hydration levels. LCP mimics the environment of the cell membrane, and thus helps membrane proteins to maintain their native conformation. Thus, membrane proteins can be crystallized by LCP-mediated aggregation (Caffrey and Cheng 1995; Nollert et al. 2002; Caffrey 2003). High resolution crystals of photosynthetic membrane proteins such as the photosynthetic reaction center from *Rhodobacter sphaeroides* (Katona et al. 2003) and the light-harvesting complex 2 from *Rhodopseudomonas acidophila* (Chiu et al. 2000) were obtained using this LCP technique.

The second technique is antibody-fragment-mediated crystallization in which the binding of Fv or Fab fragments to the target membrane protein can enlarge the hydrophilic portion of the protein, thereby providing additional surface area for molecular contacts and space for the detergent micelle. Antibody-fragment-mediated crystallization is helpful particularly for membrane proteins with a very small hydrophilic surface (Hunte and Michel 2002). Iwata successfully obtained crystals of cytochrome c oxidase using this technique (Iwata et al. 1995). The cytochrome bc(1) complex was also crystallized using this Fv-fragment-mediated technique (Hunte et al. 2000).

If a large single crystal has been obtained, but has poor or no diffraction, post-treatment may sometimes convert otherwise useless crystals into diffraction-quality crystals. Post-crystallization treatments include crystal dehydration, crystal annealing, and cross-linking (usually with glutaraldehyde) (Heras and Martin 2005). Crystals of the PSI-LHCI supercomplex from pea were improved successfully using the dehydration technique. PSI-LHCI supercomplex was first crystallized using 3.5–5% PEG6000 as a precipitant, and the resulting crystals diffracted at 4.4 Å. Prior to data collection, the PSI-LHCI crystals were transferred in three steps of increasing PEG6000 concentrations up to a final concentration of 40% over a period of 1 month. The unit cell parameters of PSI-LHCI crystals were totally changed after dehydration. The size of the unit cell shrunk by about 62%, and the molecular packing was more compact than before. The resolution of the crystal improved to 3.4 Å (Amunts et al. 2007).

Successful examples of photosynthesis membrane protein crystallization

The formation of membrane protein crystals mainly depends on the interaction and subsequent aggregation of the hydrophilic part of membrane protein molecules. According to their different packing patterns, membrane protein crystals can be divided into three types. Here we describe examples of successful crystallization of photosynthesis membrane proteins, one for each of the three types of packing patterns.

Type I crystals are stacks of 2D crystals (Fig. 2a). These crystals are usually well ordered in the 2D plane, but disordered in the third dimension. Crystals are, therefore, usually anisotropic. However, well-diffracting type I crystals, such as stacked two-dimensional crystals of pea LHC-II can also occur. Pea LHC-II crystals were grown using a hanging-drop vapor diffusion method at 20°C, using PEG-mme350 as a precipitant. The crystals were thin hexagonal plates with a typical size of $0.4 \times 0.4 \times 0.02$ mm^3 and could diffract beyond 2.2 Å (Standfuss et al. 2005). Protein molecules were packed in stacked layers containing adjacent trimers arranged in opposite orientations within one layer (PDB code 2bhw), as shown in Fig. 2b.

A high percentage of membrane protein crystals adopt type II packing (Fig. 3a). The building blocks of type II crystals are complexes of membrane proteins and detergent micelles. Crystal packing is mainly reinforced by polar

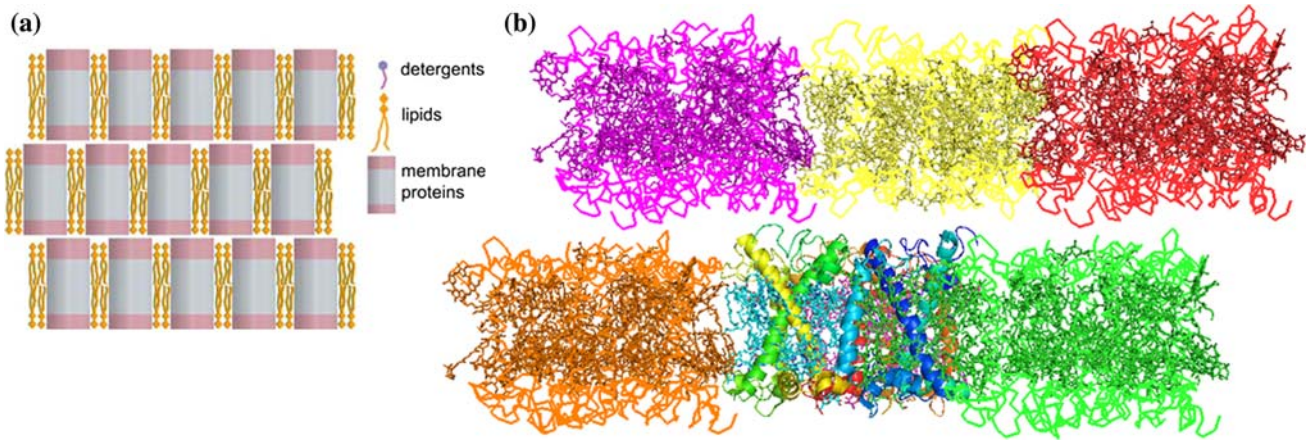

Fig. 2 Type I packing pattern of membrane protein crystals. **a** Schematic drawing of type I membrane protein crystal packing. **b** Crystal packing of Pea LHC-II (shown in *ribbon representation*) viewed parallel to the membrane plane (PDB code 2bhw). All neighboring molecules are represented as line models. (This figure was created using the PyMol program)

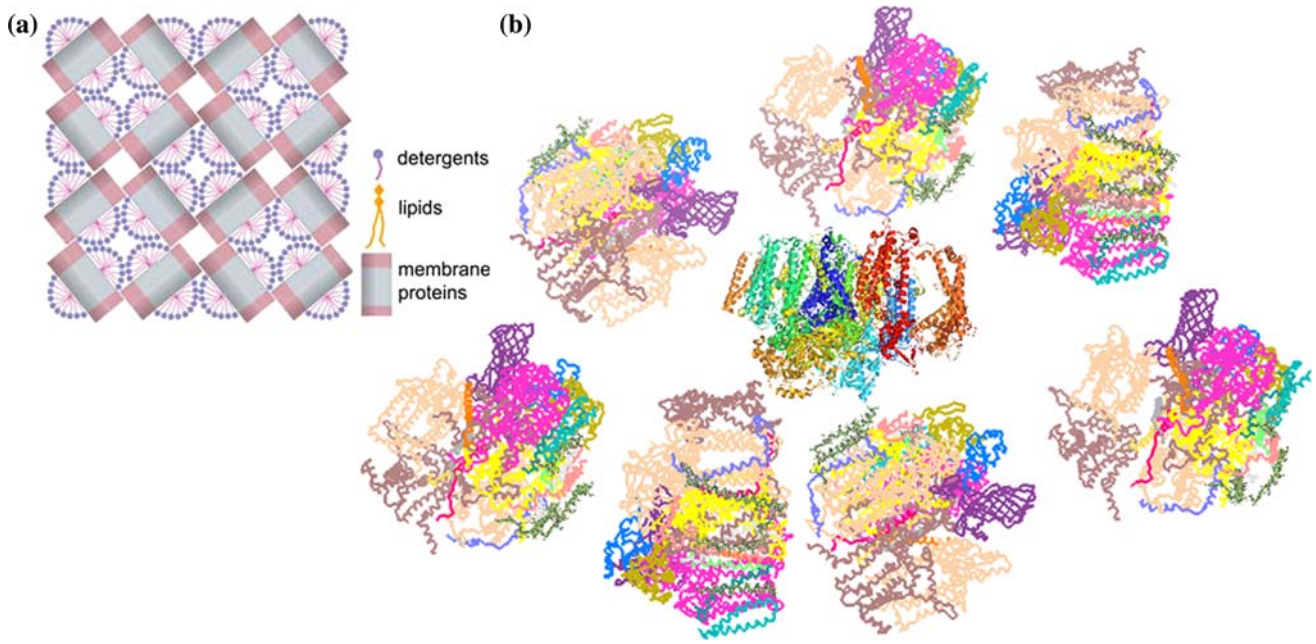

Fig. 3 Type II packing pattern of membrane protein crystals. **a** Schematic drawing of type II membrane protein crystal packing. **b** Crystal packing of photosystem II (shown in *ribbon representation*) from *Thermosynechococcus elongates* (PDB code 2axt). All neighboring molecules are represented as line models. (This figure was created using the PyMol program)

interactions between the hydrophilic surfaces of membrane proteins. Crystals of Photosystem II from *Thermosynechococcus elongates* belong to type II. The dimeric PS-II was mixed with precipitant solution and placed in the center of a capillary at 19°C (Kern et al. 2005). The PS-II crystal was a thin plate, the longest dimension of which was 0.3–0.7 mm, while the other two dimensions were only 0.1 and 0.02 mm. The crystal could diffract to 2.9 Å. The molecular packing of the crystal is shown in Fig. 3b. Interactions between protein molecules are mainly provided by the polar regions of PS-II proteins (PDB code 2axt) (Loll et al. 2005).

An example of type III crystals is that of spinach LHC-II, reported by Liu et al. (2004). Type III crystals are formed by vesicular proteoliposomes, which can be considered as closed spheres made by curled pieces of 2D membrane protein crystals. Both inner and outer surfaces of this kind of sphere are hydrophilic (Fig. 4a). The spinach LHC-II was crystallized by the sitting-drop diffusion method. Green tabular crystals were only able to form, when 2 mg/ml DGDG and 0.2% N,N-bis-(3-D gluconamidopropyl) deoxycholamide (DBC) were used as additives. The maximum size of spinach LHC-II crystals was about

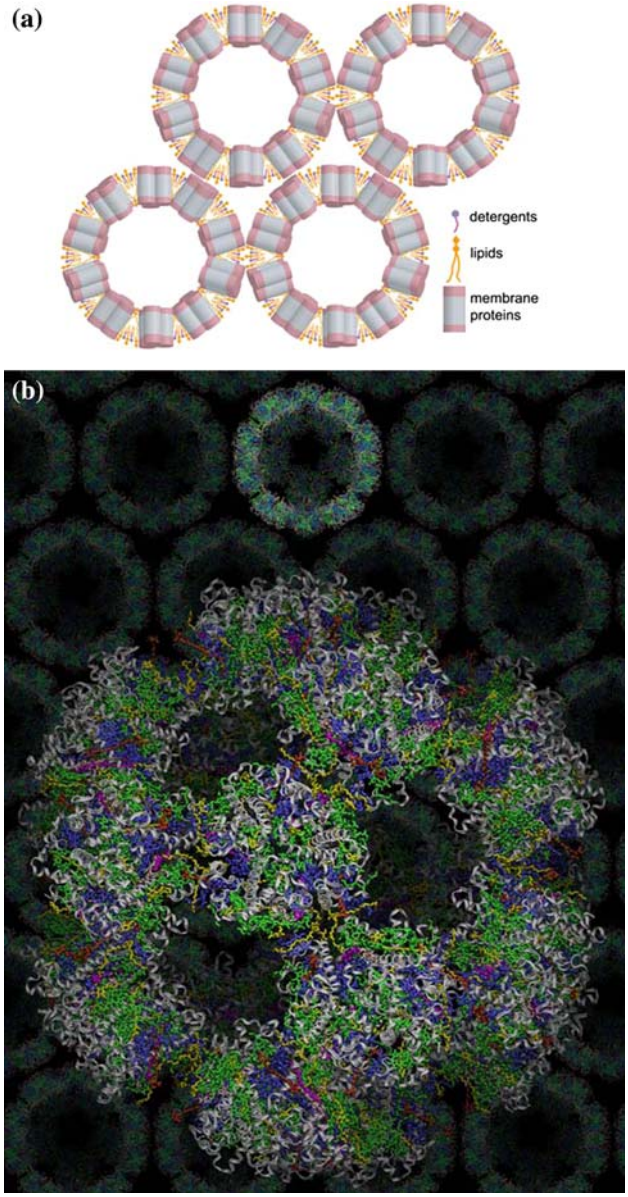

Fig. 4 Type III packing pattern of membrane protein crystals. **a** Schematic drawing of type III membrane protein crystal packing. **b** The packing of the spinach LHC-II crystal (PDB code 1rwt). Apoproteins are shown as silver ribbons. Prosthetic groups are drawn as ball-and-stick models in different colors. The background shows a portion of the crystal lattice

$0.5 \times 0.5 \times 0.05$ mm^3 and they could diffract beyond 2.2 Å. In this crystal form, 60 LHC-II monomers were assembled into a vesicular proteoliposome (PDB code 1rwt) which is the basic packing element of the crystal (Fig. 4b).

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