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SHORT COMMUNICATIONS

New techniques for membrane protein crystallization tested on photosystem II core complex of *Pisum sativum*

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Abstract The crystallization of a given protein is a hard task being even more complicated when the protein shows a hydrophobic behavior. In the case of photosynthetic proteins, the difficulty of the experiments increased due to the high light sensitivity. Aqueous solutions of photosystem II core complex (OEC PSII) of *Pisum sativum* were screened for crystallization conditions using standard crystallization methods. Crystal improvement was achieved by counter-diffusion technique in single capillaries of 0.2 mm inner diameter with a three-layer configuration. The use of this advanced crystallization technique—for the first time applied to the crystallization of membrane proteins—improves the reproducibility of the experiments allowing the initial crystal characterization, and facilitates the manipulation under light protection.

Keywords Advanced crystallization · Counter-diffusion technique · *Pisum sativum* · Photosystem II · Membrane proteins

Abbreviations

OEC PSII	Oxygen-evolving complex of photosystem II or photosystem II core complex
PSII	Photosystem II
MPDB	Membrane protein data bank
Fe	Iron
Mn	Manganese
Chl*a*	Chlorophyll *a*
PEG4000	Polyethylenglycol Mr 4000
Bis–Tris	Bis(2-hydroxyethyl)iminotris (hydroxymethyl)methane
MES	2-(*N*-morpholino)ethanesulfonic acid
MPD	2-Methyl-2,4-pentanediol
DM	*n*-dodecyl-*β*-D-maltoside

Introduction

Membrane proteins often play important roles in fundamental processes of life. Various methods such as electron microscopy, biochemical and biophysical spectroscopy methods, or electron diffraction have been used to study membrane proteins, yet to determine their structure, in most of the cases, remains problematic.

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Finding suitable crystallization condition is the main problem to solve a protein structure by X-ray diffraction techniques. The current crystallization strategy is mainly based on the screening of previously successful chemical cocktails with classical crystallization techniques based on evaporation. This strategy has been found successful for soluble globular proteins; however membrane proteins are a particular case for which the success rate applying commercial crystallization screens is much lower. The fact that many of membrane proteins are often unstable and very sensitive to temperature and light (e.g. photosynthetic proteins) along with their complicated chemical design explains why their structures are difficult to be unveiled. To date, there are only 93 structures of membrane protein deposited at the MPDB (Raman et al. 2006).

Photosynthesis is a main biological energy source for life on Earth. Photosystem II (PS II), embedded in photosynthetic (thylakoid) membranes of cyanobacteria and chloroplasts of higher plants and algae, is a multisubunit membrane-protein complex. PSII is composed of D1 and D2 proteins and α and β subunits of cytochrome b_{559} generating the reaction centre, chlorophyll-containing inner-antenna subunits CP43 and CP47, several low molecular weight subunits, pigments (chlorophylls, pheophytins and β-carotene), and cofactors as heme and non-heme binding Fe, quinones, Mn cluster, and three extrinsic proteins of the oxygen-evolving complex (OEC). All these compounds cooperate together in trapping, transferring, and modulation of solar energy to drive catalytic photo-induced oxidation of water and synthesize molecular oxygen. Catalytic mechanism of PSII has been studied using a wide range of approaches (Barber 2003; Ferreira et al. 2004; Wydrzynski and Satoh 2005), but the details of the mechanism remain unclear. Crystallographic studies of OEC PSII from thermophilic cyanobacteria have provided several medium-resolution structures from 0.38 nm to 0.30 nm resolutions (Zouni et al. 2001; Kamiya and Shen 2003; Ferreira et al. 2004; Loll et al. 2005). The main bottleneck for determining structure at higher resolution is the difficulty to obtain crystals of better diffraction quality.

Here, we report a new approach for crystallization of OEC of photosystem II core complex of *Pisum sativum* using counter-diffusion technique implemented in single capillaries.

Material and methods

Isolation of OEC PSII

OEC PSII was isolated from green pea (*Pisum sativum*) and purified according to Ghanotakis et al. (1987). The purification procedure was later improved similarly to Kern et al. (2005). The SDS-PAGE electrophoresis in a buffer system of Laemmli (1970) using 12% acrylamide resolution gel was used for analysis of isolated complexes. The protein solution was concentrated to 2–3 mg/ml of chlorophyll *a* (~1.7–2.7 mM Chl*a*) and supplemented with 1 mM $MnCl_2$ prior to crystallization trials.

Vapor diffusion crystallization

Initial crystallization screening was performed in sitting drops (Bergfors 1999; Ducruix and Giege 1999) using various commercial screens (''MembFac^{TM}''and ''Crystal Screen^{TM}'', Hampton Research, Aliso Viejo, CA, USA and ''JBScreen Crystal Screening Kits'', JenaBioscience, Jena, Germany). In parallel, crystallization conditions successfully used for photosynthetic proteins isolated from thermophilic cyanobacteria (Kuhl et al. 2000; Shen and Kamiya 2000) and higher plants (Adir 1999) were tested. Sitting drops contained 1–3 µl of protein and 2 µl of reservoir solution.

Counter-diffusion crystallization

Counter-diffusion crystallization (Garcia-Ruiz 2003) was performed in single capillaries (Lopez-Jaramillo et al. 2001). Three-layer configuration of the counter-diffusion technique (Gavira et al. 2006) consisted of 6 µl of protein solution, 3 µl of 1% buffered agarose, and 40 µl of precipitant solution in Lindeman glass capillaries (Hilgenberg GmbH, Malsfeld, Germany) with an inner diameter of 0.1 or 0.2 mm. Crystallization conditions based on PEG4000 as a precipitant and addition of detergents and salts to protein sample were optimized.

Analysis of crystals by microspectroscopy

OEC PSII crystals were analyzed by microspectroscopy using Olympus IX70 inverted microscope (Olympus, Japan) equipped with Triax320 monochromator, CCD camera (Jobin Yvon, France), and He–Cd laser emitting at 442 nm as an excitation source. This setup allows measuring fluorescence emission spectra from microscopic objects with dimension less than 300 nm. The fluorescence emission spectra were measured directly on crystals in capillaries.

Results and discussion

OEC PSII from higher plants used in crystallization trials was isolated and purified as protein complex

containing four large membrane-intrinsic subunits (CP47, CP43, D1, and D2), three extrinsic subunits (PsbO, PsbP, and PsbQ) forming OEC and low molecular protein most probably a subunit of cytochrome b-*559* (Fig. 1).

Reaction center of PSII from higher plants (spinach and pea) have been successfully crystallized by others (Adir 1999), but no crystallization attempts on OEC of PSII have been reported up to date. At first, the freshly isolated and frozen samples of the OEC PSII were extensively screened for crystallization conditions using conventional vapor diffusion technique (sitting drop) (Bergfors 1999; Ducruix and Giege 1999). Different types of precipitants, inorganic salts, various pH values, protein, and precipitant concentration ratios were tested in screening experiments. None of them produced crystalline material but precipitation, phase separation or protein denaturation. In parallel, crystallization conditions successfully used for photosynthetic proteins isolated from thermophilic cyanobacteria (Kuhl et al. 2000; Shen and Kamiya 2000) and higher plants (Adir 1999) were tested. By varying conditions of Adir (1999), green plate crystals were obtained in a sitting drop experiment at 283 K from precipitant composed of 10% PEG4000, 50 mM NaCl and 50 mM Bis–Tris, pH 7.0 (Fig. 2A). These results were never reproduced with new batch of protein and none of these crystals diffracted when tested at X13 beamline of the synchrotron DESY (Hamburg, Germany).

To optimize OEC PSII crystallization conditions and possibly improve crystal quality, we have used

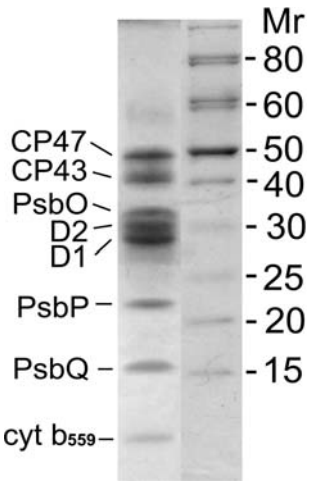

Fig. 1 SDS polyacrylamide gel electrophoresis of isolated oxygen-evolving complex of photosystem II (OEC PSII). Left line, OEC PSII sample used for crystallization, right line, protein molecular weight marker. The concentration of acrylamide in the separation gel was 12%

counter-diffusion technique (Garcia-Ruiz 2003) in single capillaries (Lopez-Jaramillo et al. 2001). This new approach, never applied to the crystallization of membrane proteins, was used to fine-tune the influence of different additives (salts, organic compounds and detergents) and pH values in a convection free environment. Small needle shaped single crystals and crystal clusters were obtained from initial screening when PEG4000 at pH 6.0–7.0 was used as a precipitant with calcium and manganese chlorides as additives. During subsequent experiments, number of factors influencing crystal nucleation and quality was identified and conditions were further optimized. The best quality OEC PSII crystals in capillaries were obtained using precipitant solution composed of 25% PEG4000, 10% MPD, 10 mM $CaCl_2$, 10 mM NaCl, 1 mM $MnCl_2$ and 150 mM MES, pH 6.5. Needle shaped crystals with dimension of about $200 \times 20 \times 20$ [μm] appeared in a week at room temperature (Fig. 2C). During our experiments, we observed that only freshly purified and non-frozen protein is suitable for crystallization as none of the experiments with frozen samples of OEC PSII yield crystals. Control vapor diffusion experiments in comparable conditions yielded precipitate or very thin needle crystals (Fig. 2B).

The main advantage of the counter-diffusion technique is that a wide range of supersaturating conditions is screening in a single experiment. We believe that the reproducibility is increased because the slight variations of protein concentration that can have a dramatic effect on vapor diffusion techniques, do not have a big influence in counter-diffusion experiments. The experimental setup, using individual capillaries for each crystallization experiment, facilitates handling under light protection. In a typical experiment, six capillaries are held in Granada Crystallization Box (Triana Sci & Tech, Granada, Spain) wrapped in aluminum foil, from which a single capillary can be extracted for observation. Sealed capillaries provide stable environment for handling and transport of crystallization experiments.

The identity of OEC PSII crystals grown in capillaries was verified by microspectroscopy. Figure 3 shows fluorescence emission spectra obtained from intact protein crystals and protein solution of OEC PSII used for crystallization. Both spectra show essentially the same features with a minor shift. The fluorescence maximum of the protein solution is 685 nm with a shoulder at 730 nm. The maximum of fluorescence emission of the crystal is shifted by 5 nm toward 690 nm. The shift is probably caused by reabsorption among highly packed protein complexes in the crystal. Since the spectra are normalized to their

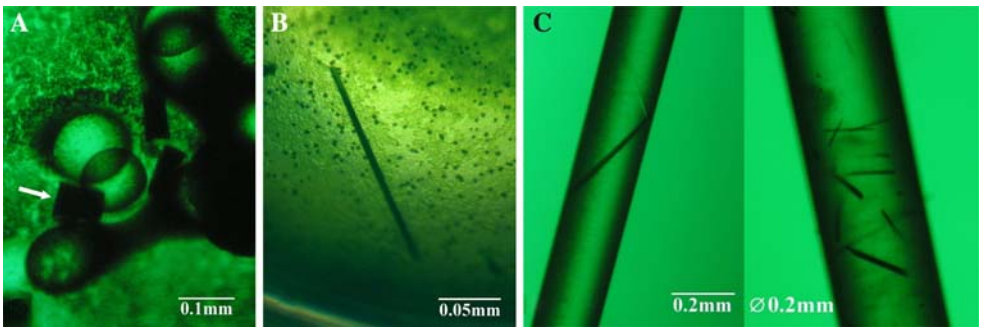

Fig. 2 Photosystem II (PSII) core complex crystals grown by vapor diffusion in sitting drops (**A**, **B**), and by counter-diffusion in a 0.2 mm inner diameter capillaries using three-layer configuration (**C**)

main peaks, the relative intensities of fluorescence bands in each spectrum are also affected by reabsorption. The number and positions of fluorescence emission peaks in the spectrum suggest absence of distorted or unbound chlorophyll molecules in OEC PSII crystals.

Best crystals grown in capillaries were also subjected to X-ray diffraction tests at the synchrotrons DESY, beamline X13 (Hamburg, Germany) and ESRF, beamlines BM16 and ID14-1 (Grenoble, France). Since the solution used for crystallization showed cryoprotective behavior, crystals when removed from the capillary could be directly flash-cooled in a nitrogen stream at 100 K. Freshly prepared crystals gave protein diffraction at low resolution (below 10 Å) while older crystals apparently suffered from aging since older crystals showed fibber diffraction patterns or did not diffract at all. We presume that crystal aging is a significant factor affecting the crystal diffraction quality, and we are currently improving both purification and crystallization procedures to produce fresh crystals measurable at a synchrotron source.

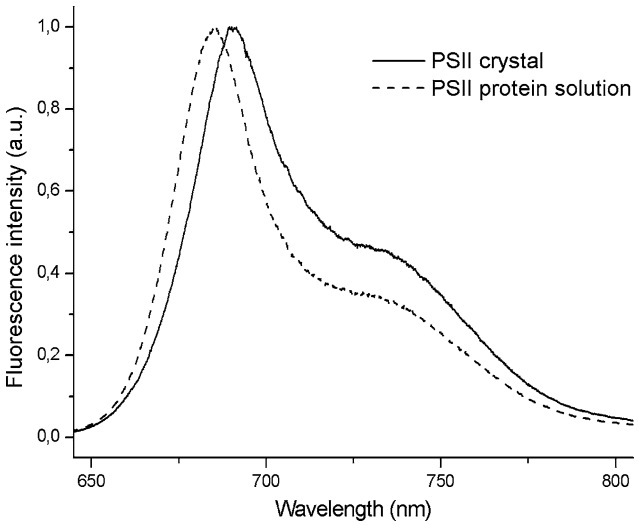

Fig. 3 Fluorescence emission spectrum of photosystem II core complex used for crystallization (dashed line) and fluorescence emission spectrum from crystals (solid line)

Conclusion

Counter-diffusion in capillaries was found to be an efficient method for optimizing crystallization conditions of sensitive membrane proteins. The use of counter-diffusion technique facilitates experimental handling under light protection and improves the reproducibility of the experiments allowing initial crystal characterization.

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