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Crystallization of the oxygen-evolving reaction centre of photosystem II in nine different detergent mixtures

Noam Adir

Department of Chemistry, The Technion, Technion City, Haifa 32000, Israel

Correspondence e-mail: nadir@tx.technion.ac.il

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Oxygen-evolving photosystem II reaction centres (RCII) isolated from both spinach and pea have been crystallized. A single crystal form grew from RCII monomers in the presence of nine different three-component mixtures of non-ionic detergents and heptane-1,2,3-triol. The crystals grew as hexagonal rods with dimensions of up to $1 \times 0.3 \times 0.3$ mm. The crystals diffracted to a maximum resolution of 6.5 Å and belong to a hexagonal space group with unit-cell parameters $a = 495$, $b = 495$, $c = 115$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The growth of a single crystal form in the presence of such a large variety of detergents suggests a very limited range of crystal lattice formation sites in the RCII complex.

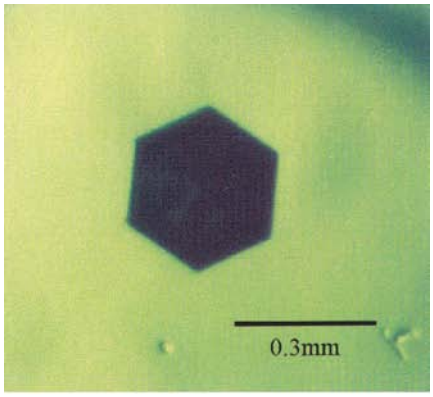
1. Introduction

Photosynthetic organisms are able to transform the energy of the sun into useful chemical energy. The photochemical reactions involved in this process occur between a variety of pigment cofactor molecules bound to large membrane-protein complexes called reaction centres (Feher *et al.*, 1989; Nitschke & Rutherford, 1991; Nugent, 1996). Of the different reaction-centre types, one of the most complex is that of photosystem II (PSII), which is found in all higher plants, green algae and cyanobacteria (Ghanotakis & Yocum, 1990; Nitschke & Rutherford, 1991; Debus, 1992, Barber & Andersson, 1994; Barber, 1998). It is the site of the initiation of linear electron flow, proton-gradient formation and oxygen evolution. It also contains an extensive and heterogeneous system of antenna pigment–protein complexes (Green *et al.*, 1991). The reaction-centre complex of PSII (RCII) contains ten known protein subunits (molecular weight ~250 kDa) organized into three subcomplexes: the reaction-centre core, two internal antenna chlorophyll-binding proteins and the oxygen-evolving complex. RCII cofactors include about 40 chlorophyll *a* molecules, two pheophytin *a* molecules, two plastoquinone molecules, one non-haem iron, four manganese ions and a membrane-bound *b*-type haem (Debus, 1992). In order to obtain information on the mechanism of RCII function, it is necessary to obtain structural information on both the cofactors and the unique protein environment surrounding each of the cofactors.

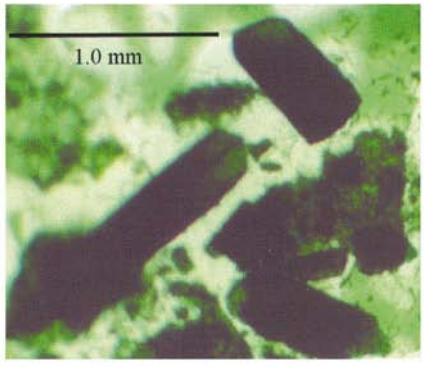
Recently, low-resolution structural information on RCII has been obtained using two-dimensional crystals in conjunction with electron microscopy and electron crystallography (Santini *et al.*, 1994; Marr *et al.*, 1996; Nakazato *et al.*, 1996; Morris *et al.*, 1997; Rhee *et al.*, 1997). From these studies, it has been suggested that RCII is dimeric in nature, with the core subcomplex of each RC making contact with a second RC core in dimer formation. The internal antenna complexes and other peripheral proteins are then suggested to surround the core. The positions of the different RCII polypeptides were also suggested in these studies.

X-ray crystallography has been used successfully to determine the structures (at different resolution levels) of a number of large membrane-bound energy-transducing complexes: the reaction centres from photosynthetic purple non-sulfur bacteria (Deisenhofer *et al.*, 1985; Allen *et al.*, 1987; Stowell *et al.*, 1997), Photosystem I (PSI; Krauss *et al.*, 1996) and the cytochrome *c* oxidase complex (Tsukihara *et al.*, 1995; Iwata *et al.*, 1995). The biggest hurdle to overcome is the growth of well ordered three-dimensional crystals of RCII. The intrinsic problems of the crystallization of membrane proteins have been well documented (Michel, 1990; Kuhlbrandt, 1992; Ostermeier & Michel, 1997). In the case of RCII there are additional problems which must be overcome. RCII is extremely heterogeneous with respect to its oligomerization state and covalent modifications (Tsiotis *et al.*, 1996; Nugent, 1996; Sharma *et al.*, 1997*a*; Zheleva *et al.*, 1998; Lyon, 1998). In addition, active RCII forms oxidative chemical species which may damage or modify the properties of the RCII complex even under extremely low light conditions (Sharma *et al.*, 1997*b*; Keren *et*

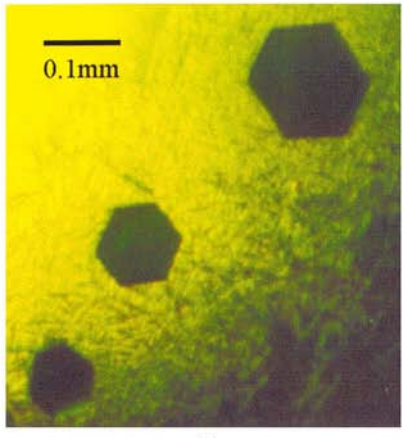
al., 1997). Various three-dimensional RCII crystals have been reported (Adir *et al.*, 1992; Fontinou *et al.*, 1993; Zouni *et al.*, 1999). In this communication, we report the crystallization of RCII in a series of nine different detergent mixtures, all of which gave the same crystal form.



(*a*)



(*b*)



(*c*)

Figure 1
RCII crystals. Panels (*a*) and (*b*), spinach RCII crystals. Panel (*c*), pea RCII crystals. The crystal in panel (*a*) was grown in detergent mix 1 (Table 1), while those in panels (*b*) and (*c*) were grown in the presence of detergent mixture 4 (Table 1). All were photographed following removal from their growth well and have a hexagonal rod-type morphology.

Table 1
Summary of detergent mixtures which promoted RCII crystal growth.

Each mixture contained 1%(*w*/*v*) of each detergent and 1%(*w*/*v*) heptane-1,2,3-triol. The mixtures were then added to the remainder of the crystallization components (see §2).

Detergent mixture	Detergent 1†	Cmc‡ [%(*w*/*v*)]	Detergent 2†	Cmc‡ [%(*w*/*v*)]
1	DM	0.009	HTG	0.85
2	DM	0.009	MEGA-8	2.5
3	DM	0.009	MEGA-9	0.84
4	Cymal-6	0.03	Cymal-3	1.6
5	Cymal-6	0.03	HTG	0.85
6	Cymal-6	0.03	MEGA-9	0.84
7	DG	0.007	OGal	0.86
8	Triton X-100	0.015	MEGA-9	0.84
9	DecM	0.09	HTG	0.85

† Cymal-3, cyclohexyl propyl-β-D-maltopyranoside; Cymal-6, cyclohexyl hexyl- β-D-maltopyranoside; DecM, *n*-decyl-β-D-maltopyranoside; DG, dodecyl-β-D-glucoside; DM, *n*-dodecyl-β-D-maltopyranoside; HTG, 1-*s*-heptyl-β-D-thioglucopyranoside; MEGA-8, octanoyl-*N*-methylglucamide; MEGA-9, nonanoyl-*N*-methylglucamide; OGal, octyl-β-D-galactoside. ‡ Cmc, critical micelle concentration.

2. Materials and methods

2.1. Isolation of RCII

The method for isolation of RCII from spinach is based on the procedure described in Adir *et al.* (1992); modifications to this procedure will be discussed elsewhere (Adir, Cheredman & Lerner, in preparation). Pea seedlings were grown on vermiculite for 10–12 d in a hothouse. Isolation of RCII from the pea photosynthetic membranes was performed using the same procedure as for spinach. The isolated RCII was monomeric as deduced from size-exclusion HPLC analysis (Adir, Cheredman & Lerner, in preparation). The final purified protein samples were dialysed against 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.0 in the presence of 5 m*M* NaCl, 5 m*M* $CaCl_2$ and 0.025%(*w*/*v*) dodecyl-β-D-maltoside (DM) and concentrated by ultrafiltration to 70–100 mg ml^{-1} prior to crystallization trials.

2.2. Crystallization in the presence of detergent mixtures

Crystallization was performed in sitting-drop type format (Cryschem, Charles Supper). The concentrated protein was incubated with mixtures of added detergents (Table 1) and additives used in the crystallization trial, followed by addition of the precipitant polyethylene glycol 4000 [PEG 4K; 4%(*w*/*v*)]. The final drop contained 12–14 mg ml^{-1} protein (2 mg ml^{-1} chlorophyll *a*), 1%(*w*/*v*) of each of the detergents (Anatrace Inc.) from one of the detergent mixtures (Table 1) and 1%(*w*/*v*) heptane-1,2,3-triol (HT; Sigma), 1 m*M* $MnCl_2$ and 50 m*M* bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis–Tris) pH 7.0. The reservoir contained 10% PEG 4K and 0.1 *M* NaCl in 50 m*M* Bis–Tris. Crystals grew in 2–4 weeks at 292 K and in 3–6 weeks at 277 K.

2.3. Analysis of protein composition of RCII crystals

RCII crystals were pipetted out of their growth wells and washed twice in an artificial mother liquor (18% PEG 4K in 50 m*M* Bis–Tris buffer pH 7.0) by gentle mixing followed by a short centrifugation. The pellet of crystals was then solubilized in 10–20 µl of buffer *A* with 0.05% DM. The crystals dissolved immediately and the resulting solution was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970).

3. Results and discussion

Well formed three-dimensional crystals could only be obtained by utilizing detergent mixtures. These mixtures contained two non-ionic detergents of different critical micelle concentrations (cmc) and the amphiphile HT (Michel, 1990). The first mixture was described by Adir *et al.* (1992). Attempts at improving crystal diffraction quality yielded an additional eight detergent mixtures which promoted crystal growth (Table 1). All of these conditions promoted the growth of hexagonal rods. In a very large number of crystallization trials (>50), where other detergent mixtures or single detergents (with or without HT) were used, only amorphous precipitate or thin needle-shaped crystals were obtained. Figs. 1(*a*) and 1(*b*) show spinach RCII crystals with dimensions of up to 1 × 0.5 × 0.5 mm, while Fig. 1(*c*) shows similar crystals of pea RCII. The crystals typically grew in the presence of amorphous precipitate (Fig. 1*b*) and/or films. The amorphous precipitates and films were completely insoluble, preventing analysis of

the RCII proteins surrounding the crystals in these cases as well as in those cases where no crystals were obtained.

The protein composition of the crystals was analysed by SDS–PAGE on a 10–20% polyacrylamide gel in the presence of 6 *M* urea (Fig. 2, lane 2). The electrophoretic pattern shows eight of the ten RCII poypeptide subunits and is identical to that of RCII prior to crystallization (lane 1). Two additional small polypeptides are not resolved on this gel. It thus appears that the detergent mixtures do not affect the integrity of the RCII complex.

The X-ray diffraction pattern of RCII crystals were examined on a Rigaku R-AXIS IIc diffractometer and showed diffraction to about 8 Å. 1.0° oscillation frames were collected and analyzed using the *DENZO* autoindexing software package (Otwinowski, 1993). The crystals best fit a hexagonal space group, with approximate unit-cell parameters $a = 495$, $b = 495$, $c = 115$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Test exposures using the synchrotron source at ESRF Grenoble (beamline ID02B) indicated that RCII crystals diffract anisotropically to a maximum resolution of 6.5 Å along one axis and to 9 Å along the second axis (Fig. 3). The crystals degraded very rapidly in the X-ray beam. Experiments are under way to improve radiation stability by cryo-crystallographic methods.

The unit-cell volume is approximately 2.4×10^7 Å^3. Typically, the ratio of unit volume to molecular weight (V_m) in crystals of soluble proteins is in the range 2.5–3.5 Å^3 Da^{-1} (Matthews, 1968). It has been seen in cases of crystallized membrane proteins that the ratio is greater than that of soluble proteins, in the range of 4–6 Å^3 Da^{-1} (Adir *et al.*, 1996). While there is some uncertainty about the actual space group of the crystallized RCII, in order to be in the correct range the crystal unit cell should contain 18–24 RCII monomers. The monomers could be arranged in either six trimers or six tetramers (V_m = 5.3 or 4.1, respectively).

The appearance of the same crystal form in the presence of such a wide range of detergent mixtures was unexpected. Typically, major modifications to a crystallization mixture (such as use of different detergents) result in either no crystal growth or the growth of different crystal forms. The results presented here suggest that in the case of RCII, sites for hydrophilic interactions available for crystal lattice formation may be very limited, resulting in the single crystal form. The detergent mixtures in Table 1 may all form an annulus (Michel, 1990) of a size compatible with the crystal lattice, while other combinations may be of an incorrect size. It is also possible that RCII is unstable in some detergents; however, in the cases where no crystals were obtained, the insolubility of the precipitate precluded further analysis. A series of biochemical and biophysical measurements aimed at characterizing the crystallized RCII and its interaction with detergent mixtures have been performed, and will be described elsewhere (Adir, Cheredman & Lerner, in preparation).

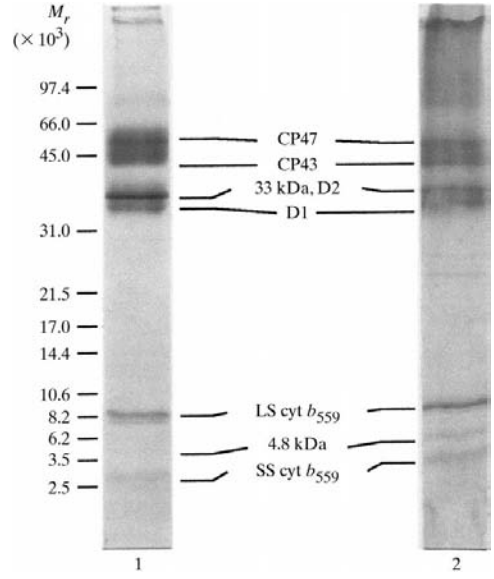


Figure 2
Electrophoretic analysis of spinach RCII before and after crystallization. Lane 1, purified RCII in 0.025% DM. Lane 2, RCII from solubilized crystals. Both lanes contained 1.5 µg chlorophyll (~10 µg protein). In lane 2 (crystallized RCII) aggregated material is seen in the upper part of the lane. M_r, molecular-weight standards, prepared by mixing a low-range protein mixture (97.4–14.4 kDa; Bio-Rad) with a peptide marker kit (17–2.5 kDa; Pharmacia)

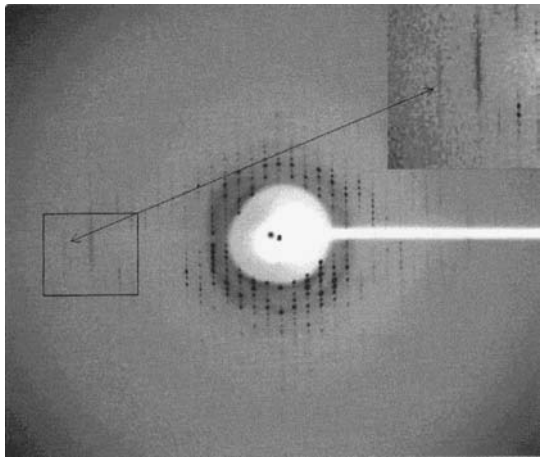


Figure 3
1° oscillation diffraction pattern from a spinach RCII crystal grown in detergent mixture 4 (Table 1). The image was recorded on a MAR Research imaging-plate detector system at a crystal-to-film distance of 400 mm and with an exposure time of 2 s at beamline ID0B2 at ESRF, Grenoble. The arrow indicates diffraction spots at ~6.5 Å (inset). Diffraction perpendicular to the highest resolution spots is at about 9 Å.

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