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Purification, characterisation and crystallisation of photosystem II from *Thermosynechococcus elongatus* cultivated in a new type of photobioreactor

J. Kern[a], B. Loll[b], C. Lüneberg[a], D. DiFiore[a], J. Biesiadka[b], K.-D. Irrgang[a], A. Zouni[a,*]

[a]*Max-Volmer-Laboratory for Biophysical Chemistry and Biochemistry, Technical University Berlin, 10623 Berlin, Strasse des 17. Juni 135, Germany*
[b]*Institute of Crystallography, Free University Berlin, 14195 Berlin, Takustr. 6, Germany*

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Abstract

The thermophilic cyanobacterium *Thermosynechococcus elongatus* was cultivated under controlled growth conditions using a new type of photobioreactor, allowing us to optimise growth conditions and the biomass yield. A fast large-scale purification method for monomeric and dimeric photosystem II (PSII) solubilized from thylakoid membranes of this cyanobacterium was developed using fast protein liquid chromatography (FPLC). The obtained PSII core complexes (PSIIcc) were analysed for their pigment stoichiometry, photochemical and oxygen evolution activities, as well as lipid and detergent composition. Thirty-six chlorophyll *a* (Chl*a*), 2 pheophytin *a* (Pheo*a*), 9± 1 β-carotene (Car), 2.9±0.8 plastoquinone 9 (PQ9) and 3.8±0.5 Mn were found per active centre. For the monomeric and dimeric PSIIcc, 18 and 20 lipid as well as 145 and 220 detergent molecules were found in the detergent shell, respectively. The monomeric and dimeric complexes showed high oxygen evolution activity with 1/4 O_2 released per 37–38 Chl*a* and flash in the best cases. Crystals were obtained from dimeric PSIIcc by a micro-batch method. They diffract synchrotron X-rays to a maximum resolution of 2.9-Å, resulting in complete data sets of 3.2 Å resolution.

Keywords: Photosystem II; Crystallisation; *Thermosynechococcus elongatus*; Cofactor composition; Oxygen evolution activity; Cell cultivation

1. Introduction

Photosystems I and II (PSI, PSII) are two large multi-subunit protein complexes embedded in the thylakoid membrane of cyanobacteria, algae and higher plants being responsible for light-driven charge separations in oxygenic photosynthesis (for recent review on photosynthesis see [1]). PSII consists of four large membrane-intrinsic subunits (D1, D2, CP43, CP47), several smaller membrane intrinsic (including PsbE and PsbF, constituting cyt *b*-559) and three extrinsic subunits (PsbO, PsbU, and PsbV in cyanobacteria and PsbO, PsbP and PsbQ in green algae and higher plants). The organic cofactors bound to PSII include chlorophyll *a* (Chl*a*), pheophytin *a* (Pheo*a*), plastoquinone 9 (PQ9), haem, and carotenoids (mainly β-carotene) (Car). For light-driven charge separation and subsequent electron transfer, PSII utilizes a chain of cofactors arranged across the membrane and bound to subunits D1 and D2. The primary electron donor of PSII named P680 is probably formed by four Chl*a* molecules (P_{D1}, P_{D2}, Chl_{D1}, Chl_{D2}) according to the multimer model [2,3] and located close to the lumenal side of the thylakoid membrane. After formation of $P680^{+\cdot}$ the released electron is passed via a Chl*a* and one Pheo*a* to the electron acceptor Q_A (PQ9) at the stromal side. The electron is further transferred to Q_B (a second PQ9) which, after two electron transfers, leaves PSII as doubly reduced and protonated plastoquinol. The catalytic site for water oxidation is located at the lumenal side, probably composed of 4 Mn ions, 1 Ca^{2+}, and 1 Cl^-. Several

* Corresponding author. Fax: +49 30 31421122.
E-mail address: zouni@phosis1.chem.tu-berlin.de (A. Zouni).

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amino acid residues mostly from D1 and probably CP43 ligate these inorganic cofactors [4–7].

In the last years three groups published crystal structures of PSII from the thermophilic organisms *Thermosynechococcus elongatus* and *T. vulcanus* at medium resolution of 3.8 to 3.5 Å [6,8–10]. Here we describe in detail the protocol used for purification and crystallisation of PSII leading to better quality of the crystals and higher resolution of X-ray diffraction [7].

For isolating and purifying PSII core complexes (PSIIcc) from *T. elongatus*, several procedures were published so far [11–22]. In order to obtain large amounts of crystallisable PSIIcc, it was necessary to develop a fast and large-scale purification method. Previously published protocols either used very time-consuming density gradient centrifugations appropriated for separating only limited amounts of protein or employed a mixture of detergents for solubilisation. The approach described here is different as it uses a single type of detergent (*n*-dodecyl-β-D-maltoside (βDM)) and fast protein liquid chromatography (FPLC) for purifying the PSIIcc.

2. Materials and methods

2.1. Cell culture, protein purification and crystallisation

T. elongatus cells were grown at 56 °C in a PBR25 photobioreactor (IGV Potsdam, Germany and Sartorius-BBI Systems, Melsungen, Germany) using a volume of 32 l under continuous circulation of the medium. The culture was illuminated by 2 to 12 lamps (Fluora 550 Im L18W/77, Osram, Germany) and adjusted to pH 7.8 by automatically adding CO_2. The light intensity and flow rate were regulated following the cell density in the culture.

The culture was started either by inoculating a thawed cell stock or by using freshly grown cells from a previous cultivation. After a maximum of eight culture cycles, the following culture was again initiated from frozen cell stock. Cell cultivation was carried out in Castenholz Medium D [23] at an initial OD of 0.15. Cells were harvested after 7 days at a final OD of 1.3. Thylakoids were prepared from cells after lysozyme digestion, cell disruption using a Yeda press at 30 atm nitrogen pressure and osmotic shock, as described previously [11]. Thylakoid membranes were suspended in buffer MGCM (20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–NaOH, pH 6.5, 25% (v/v) glycerol, 20 mM $CaCl_2$, 10 mM $MgCl_2$) to 1.8 mM Chl*a* and solubilized using 0.5% to 0.6% (w/w) βDM, 1 mM phenylmethanesulfonyl fluoride (PMSF) in MGCM for 20 min at RT in the dark. Solubilized protein was separated from membrane fragments by centrifugation for 100 min at 50 000 rpm in a 70 Ti rotor (Beckman Instruments, USA) at 4 °C. All further steps were conducted at 6 °C in the dark or under dim green light.

Buffers used for protein chromatography were mixed from buffer A (40 mM MES–NaOH, pH 6.0, 20 mM $CaCl_2$, 0.02% (w/w) βDM, 5% (w/w) glycerol) and buffer B (as buffer A including 50 mM $MgSO_4$). Protein extract derived from about 80 g of cells was diluted with the same volume of 24% B and applied to preparative chromatography on an ÄktaFPLC System (Amersham Biosciences, Germany). The first column was 50 mm in diameter and 300 mm in length (Kronlab, Germany), packed with Toyo Pearl DEAE 650 S (TosoHas, PA, USA), preequilibrated with 24% buffer B. After sample loading the column was washed at constant salt concentration (24% B, 5 column volumes (CV)) followed by an isocratic step to 50% B and a linear gradient from 50% to 80% B in 3 CV at a flow rate of 20 ml/min. The eluate was fractionated and the PSII containing peak eluting at 50–65% B was pooled. The pooled peak was diluted with two volumes of buffer A and loaded onto the second Toyo Pearl DEAE 650 S column (25 mm diameter, 350 mm length; Kronlab), preequilibrated with 24% B. The column was run at a flow rate of 7 ml/min for 10 h. A washing step of 5 CV with 24% B was followed by a linear gradient from 24% to 80% B in 7 CV. The eluate was fractionated and the two main peaks were separately pooled and concentrated in an Amicon stirring cell using a Biomax 100 membrane (Millipore, MA, USA). For further concentration to volumes <1 ml, Ultra Free 100 concentrators (Millipore) were used in a centrifuge at 3000 rpm at 4 °C. The concentrated protein solution was stored either in buffer A with 25 mM $MgSO_4$ in liquid nitrogen (in case of monomeric PSIIcc) or was further purified by crystallisation (dimeric PSIIcc).

2.2. Crystallisation and X-ray diffraction analysis

Immediately after concentration, the dimeric PSIIcc was adjusted to a Chl*a* concentration of 0.74 mM by dilution with buffer C (100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)–NaOH, pH 7.0, 5 mM $CaCl_2$, 0.03% (w/w) βDM). The protein solution was slowly mixed with the same volume of precipitation buffer D (100 mM PIPES–NaOH, pH 7.0, 5 mM $CaCl_2$ and 15% (w/w) polyethylenglycol (PEG) 2000). After incubation for 1 h, the amorphous precipitate was centrifuged and the pellet was redissolved in buffer C yielding a concentration of 0.74 mM Chl*a*. This precipitation step was repeated twice with the same solution and decreasing concentration of PEG 2000. In the third step, micro-crystals were obtained overnight, which were redissolved in buffer C to yield a final Chl*a* concentration of 4 mM.

For batch crystallisation, glass capillaries were used (75 mm length, 1.5 mm inner diameter; Hirschmann, Germany). The dimeric PSIIcc (4 mM Chl*a* in buffer C) was mixed under slight vortexing with the same volume of precipitant solution containing 6% to 8% PEG 2000, 100 mM PIPES–NaOH, pH 7.0, 5 mM $CaCl_2$. Five microlitres of the obtained solution were placed in the centre of a capillary.

The ends were sealed with sealing plaster and the capillaries were kept in the dark at 19 °C for 2 to 5 days.

For X-ray diffraction experiments, PSII crystals were stepwise transferred to buffer C supplemented with 10% (w/w) PEG 2000, 30% (w/w) glycerol and flash-cooled in a nitrogen gas stream at 100 K. X-ray diffraction experiments were performed at 100 K at the following synchrotron facilities: ESRF (Grenoble), SLS (Villigen), DESY (Hamburg), BESSY II (Berlin). The beamlines were equipped with a nitrogen gas cryostat (Oxford Cryosystems, UK) and either a Q4 CCD detector (ADSC, USA) or a MAR CCD detector (Marresearch, Germany). Diffraction images were processed with DENZO [24] and scaled with SCALEPACK [24].

2.3. Analytical methods

The Chl*a* concentration of PSIIcc solutions was determined at RT after extraction in 80% (v/v) acetone using $\varepsilon_{(663.6–750)}$=76.8 mM^{-1} cm^{-1} [25] and measuring absorptions at 664 and 750 nm in a Cary 50 spectrophotometer (Varian, CA, USA). UV–VIS spectra were recorded in the same spectrophotometer with 0.5 nm step size, 600 nm/min scan speed; samples were diluted with buffer A or used organic solvent, respectively.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done with a Phast-System (Amersham) using precast SDS-HD gels (Amersham). Gels were run and silver stained following the protocol recommended by the manufacturer. Samples were mixed 1:1 with sample buffer (20 mM Tris/HCl, pH 6.8, 2 mM EDTA, 5% SDS, 0.2% DTT) resulting in a Chl*a* concentration of 20–60 μM. After incubating at 56° for 15 min, 1 μl of the sample was applied per lane (20–60 pmol Chl*a*). Low molecular weight and peptide marker kits (Amersham) were used for determining the apparent molecular masses.

For size exclusion chromatography, an Äkta FPLC chromatography system (Amersham) equipped with a Superose 6 HR10/30 column (Amersham) was used. The column was run in buffer A supplemented with 25 mM $MgSO_4$, at a flow rate of 0.5 ml/min. UV-absorption was monitored at 280 nm.

CD spectra were measured using a Jasco J-500 spectropolarimeter (Jasco, Japan). Response time, sensitivity and scan speed were 0.5 s, 50 mdeg/FS and 20 nm/min, respectively. A freshly prepared solution of D-camphor-10-sulfonate was used for calibration. The band width was 1.0 nm and the resolution 0.1 nm. The samples were diluted to a concentration of 50 μM Chl*a* in buffer A supplemented with 25 mM $MgSO_4$ and measured in a 2-mm-wide quartz cuvette. The temperature was varied between 25 and 95 °C. For each temperature, 10 scans were averaged in the far-UV region from 200 to 260 nm.

Oxygen evolution measurements were conducted at RT, using a home built Clark type electrode [26]. Excitation was either performed with repetitive 1 Hz flashes from a xenon flash lamp or with saturating continuous white light from a tungsten lamp passed through a heat filter. The buffer used contained 20 mM MES–NaOH, pH 6.4, 5 mM $CaCl_2$, and the sample concentration was diluted to 20–50 μM Chl*a*. Artificial electron acceptors added were either 2 mM $K_3[Fe(CN)_6]$ and 0.2 mM phenyl-*p*-benzoquinone or 2 mM 2,6-dichloro-*p*-benzoquinone. The electrode was calibrated using air-saturated and nitrogen-saturated water at atmospheric pressure.

Q_A was quantified by measuring flash induced transient absorption changes at λ=325 nm. Flashes were provided by a xenon flash lamp equipped with an RG-630 filter at 1 Hz repetition rate. Measuring light was provided by a 250 W tungsten lamp; transient absorption differences were detected using a photomultiplier and recorded by a transient recorder (Tektronix, OR, USA). Measurements were conducted at RT in a 2-mm path length sample cuvette in 45° orientation, giving an effective optical path length of 2.4 mm. Sample concentration was 40 μM Chl*a* in 20 mM MES–NaOH, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.2 mM 2,6-dichloro-*p*-benzoquinone, 2 mM $K_3[Fe(CN)_6]$. For Q_A quantification an absorbance difference coefficient of $\Delta\varepsilon(Q_A/Q_A^-)$=13,000 M^{-1} cm^{-1} at 325 nm [27] was used.

For carotenoid quantification, spectra of pigments extracted with 80% (v/v) acetone were recorded and a spectrum of pure Chl*a* (Fluka, Germany) in the same solvent was substracted from the normalised spectra. From the resulting difference spectrum, the Car content was determined using ε_{454} =14.4 mM^{-1} cm^{-1} [28].

For reverse phase high pressure liquid chromatographic (RP-HPLC) analysis of pigments, extracts from protein equivalent to 0.06 μmol Chl*a* in either 90% (v/v) acetone or 100% methanol were loaded on a Nucleosil C18 column (Knauer, Germany) connected to an Äkta HPLC System (Amersham). Eluent was 90% (v/v) methanol, 10% (v/v) THF at a flow rate of 1 ml/min; detection was done simultaneously at 255, 410 and 665 nm. For determining the Chl*a*/Pheo*a* ratio independent of the extinction coefficients, the UV–VIS spectrum of pure Chl*a* (Fluka) in 90% (v/v) methanol, 10% (v/v) THF was recorded. After measuring the spectrum, the Chl*a* was converted to Pheo*a* by adding 1-μl 3 N HCl/ml to the sample solution, and the spectrum of the obtained pure Pheo*a* was subsequently recorded. The extinction ratio of Chl*a*/Pheo*a* at 410 nm was determined for chromatographically quantifying both chromophores. The PQ9/Pheo*a* ratio was calculated using extinction coefficients of ε_{255}=15.2 mM^{-1} cm^{-1} for PQ9 [29] and ε_{410}=114.3 mM^{-1} cm^{-1} for Pheo*a* [28], respectively.

Lipid and βDM content was investigated using 2-d thin layer chromatography (TLC). The lipids were extracted as described in Ref. [30]. 2-d TLC was conducted using TLC silica 60 plates (VWR, Germany) and a solvent system of 65:25:4 (v/v/v) chloroform/methanol/water in the first dimension and 85:15:10:3.5 (v/v/v/v) chloroform/methanol/acetic acid/water in the second dimension. For lipid quantification, spots were scraped out of developed TLC

plates after short iodine staining and fatty acid methyl esters were prepared and quantified by gas chromatography (GC) as in Ref. [31]. For colorimetric quantification, βDM spots were visualized using iodine, scraped out of the TLC plate, extracted two times with 250 μl methanol/chloroform 1:1 (v/v), evaporated to dryness and redissolved in 50 μl water. Two-hundred fifty microlitres of 0.5% (w/v) phenol was added to the obtained sample solution and mixed with 600 μl concentrated H_2SO_4. Samples were incubated for 30 min and formed furfural derivatives were detected by measuring their absorbance at 490 nm [32].

The Mn content was determined by atom absorption spectroscopy using the graphite furnace technique in an AAS800 spectrometer (Perkin Elmer, Germany). Mn standard solution (Fluka) and samples were diluted in 0.2% (v/v) HNO_3. Measurements were carried out in the presence of a matrix modifier (5 μg $Mg(NO_3)_2$) at 279.5 nm using a slit width of 0.2 nm and the temperature program recommended by the manufacturer. Data handling and evaluation was done with AAwinlab (Perkin Elmer).

3. Results

3.1. Cell growth and isolation of PSII

Using the novel PBR, photobioreactor light intensity and flow rate were regulated following the cell density of the culture (for details see Materials and methods). A cell density of 1.3 OD was obtained within 7 days and about 80–100 g cells (wet weight) could be harvested from 32 l of culture.

The detailed procedure developed for solubilising and purifying PSIIcc is described in Materials and methods. Thylakoid membranes were solubilized with βDM and the crude extract was purified in a two-step chromatograpic procedure, using Toyopearl DEAE 650 S. In the first step, the crude protein extract was quickly separated into three main fractions: (1) phycobilisomes, (2) PSII and (3) PSI. As fraction (2) still contained some phycobilisomes, traces of PSI and ATPsynthase, this fraction had to be further purified. For this second chromatography the same column material and buffer system were used as before, but using different column geometry, flow rate and protein loading (see Materials and methods). Thereby fraction (2) from the first chromatography was further separated into phycobilisomes and ATP synthase, monomeric PSIIcc, dimeric PSIIcc and PSI. Fig. 1 shows a typical chromatogram of the second step of the preparative FPLC run.

Following this procedure, we got crystallisable protein material within 24 h starting from thylakoid membranes. Typically a total yield of 140 mg protein was obtained for dimeric and 90 mg for monomeric PSIIcc after the second column chromatography and concentration of the samples. The total yield of PSIIcc (monomeric and dimeric) after column chromatography varied from 2% to 10% of the total Chl*a* content in the starting extract, the mean value of 35 preparations was 5.7±2%. After the second chromatography step, the PSIIcc monomer/dimer ratio was found to be 0.7±0.3, but sometimes even ratios of 0.1 or up to 1.5 were observed.

3.2. Sample characterisation

Fig. 2a depicts UV–VIS spectra of crude extract, the PSII fraction after the first column chromatography, and monomeric and dimeric PSIIcc fractions after the second one. Contaminations by PSI and phycobilisomes, visible in the crude extract, are strongly reduced after the first column

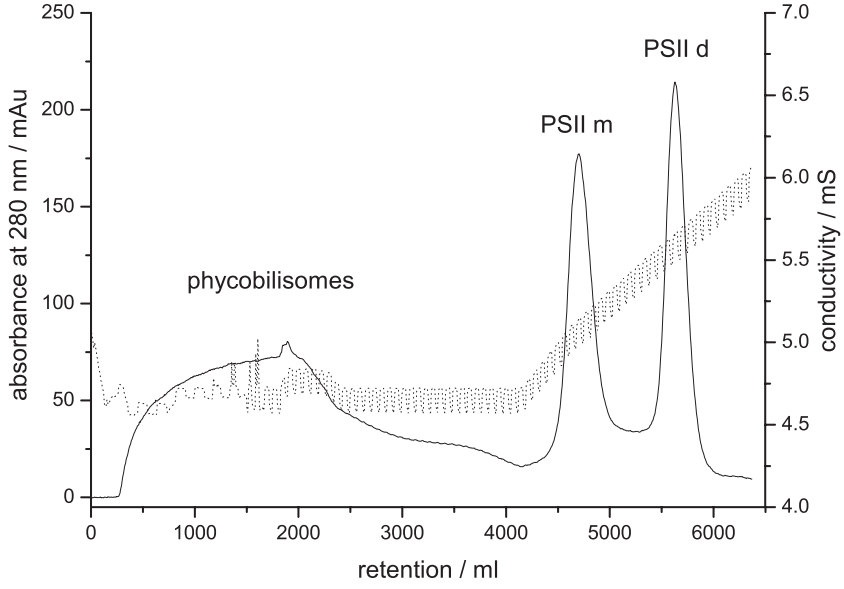

Fig. 1. Preparative anion exchange chromatography of PSIIcc. The chromatogram shows the second chromatographic step for separation of monomeric (m) and dimeric (d) PSIIcc. Solid line: absorption at 280 nm; dotted line: conductivity. Details as described in Materials and methods.

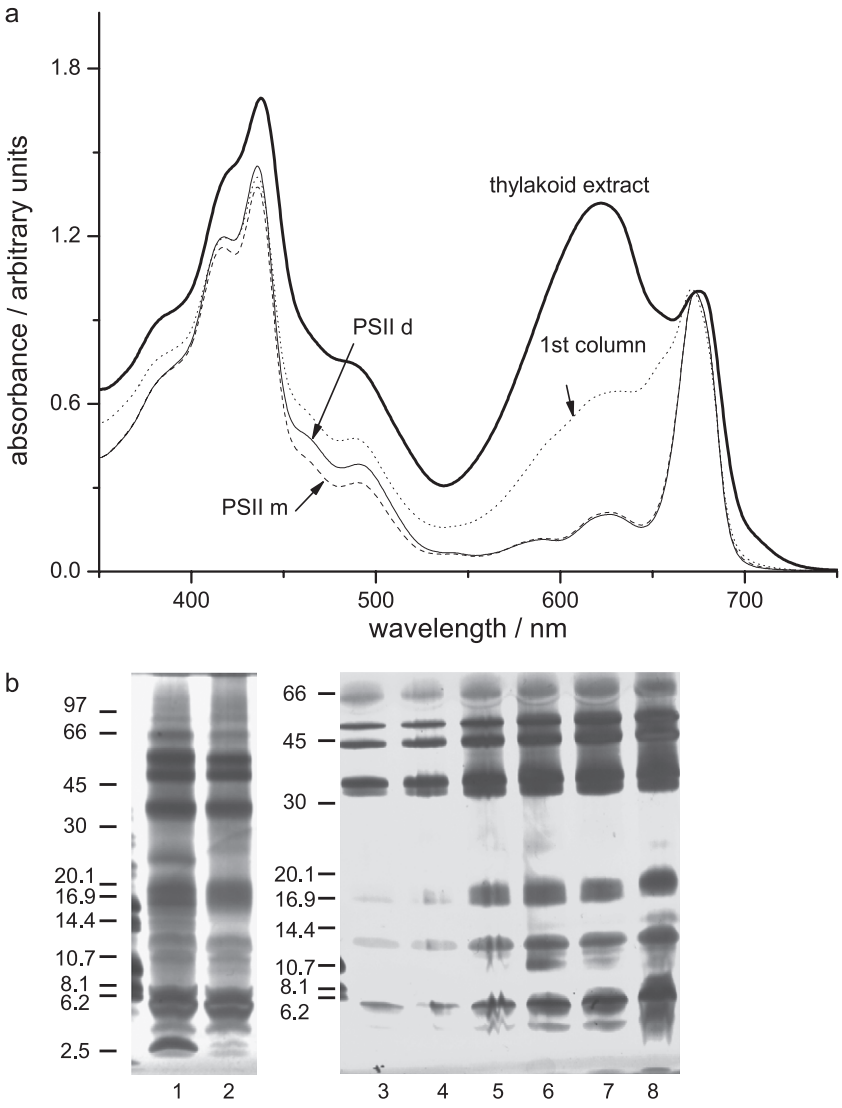

Fig. 2. (a) UV–VIS spectra of crude extract (thick solid line), PSII fraction after the first column chromatography (dotted line), monomeric (dashed line) and dimeric PSIIcc (solid line) after rechromatography, normalised to absorption at 680 nm. (b) Silver-stained SDS-HD-PAGE of PSII. Lane 1: monomeric PSIIcc (40 pmol Chl*a*); lane 2: dimeric PSIIcc (40 pmol Chl*a*); lanes 3–8: redissolved crystals of PSIIcc at increasing concentrations (20, 25, 30, 40, 50, 60 pmol Chl*a*/lane), positions of marker bands and corresponding molecular weights are indicated on the left side of both silver stained gels.

chromatography. After rechromatography, monomeric and dimeric PSIIcc fractions show nearly identical spectra (except for slight variations in the Car content) without any detectable PSI contamination.

For further characterizing the molecular composition of the samples SDS-PAGE was carried out. CP47, CP43, D1, D2, PsbO, PsbU, PsbV, PsbE, PsbH and several smaller polypeptides were identified in monomeric as well as in dimeric PSIIcc (see Fig. 2b, lanes 1 and 2). The same pattern was obtained for redissolved crystals (Fig. 2b, lanes 3–8), indicating that no changes of the polypeptide composition occurred upon crystallisation. This was supported by matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectral analyses of the same samples [33].

Analysis of both PSII fractions by size exclusion chromatography clearly showed different retention times for both samples, supporting the assignment to monomeric and dimeric PSIIcc (Fig. 3a) (for details of molecular weight determination, see Ref. [34]).

The stability of the monomeric and dimeric PSIIcc was studied by CD spectroscopy as a function of temperature. The CD spectra did not significantly change in the temperature range of 20 to 50 °C, however, above 60 °C dramatic spectral changes were observed. From the change of ellipticity at 220 nm the phase transition temperature for monomeric and dimeric PSIIcc was determined to be 68 and 71 °C, respectively (Fig. 3b).

For analyzing the water splitting and photochemical activities of the PSIIcc, the following experiments were done: (a) measuring oxygen evolution under continuous light as well as single flash excitation. Typical values obtained using both methods are shown in Table 1. (b)

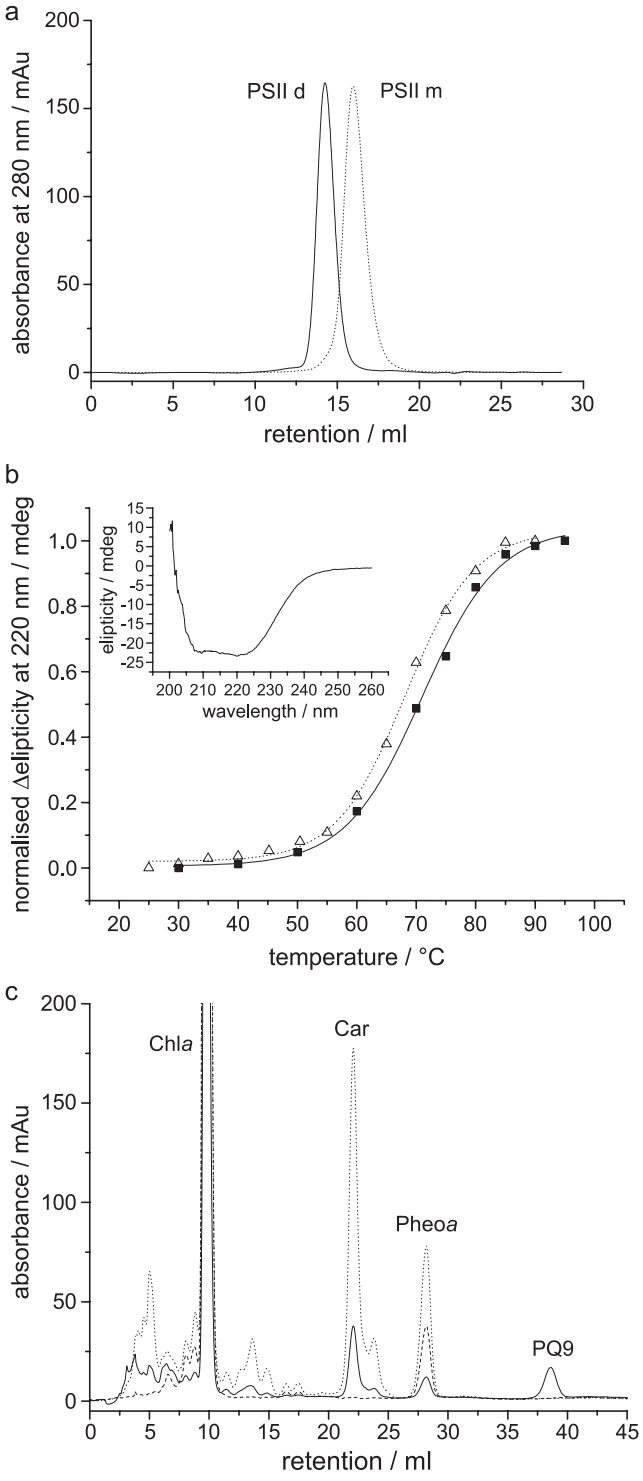

Fig. 3. (a) Gel permeation chromatography of monomeric (dotted line) and dimeric (solid line) PSIIcc using a Superose 6 column; details described in Materials and methods. (b) Denaturation of PSII induced by a temperature increase as observed by CD spectroscopy. Change of ellipticity at 220 nm normalised to the maximum value for monomeric (open triangles) and dimeric (solid squares) PSIIcc between 25 and 90 °C is shown. In the insert the CD spectrum of dimeric PSIIcc at 30 °C is depicted. (c) Reverse phase HPLC chromatogram of monomeric PSIIcc on a Nucleosil C18 column (see Materials and methods for details); detection at 255 nm (solid), 410 nm (dotted) and 665 nm (dashed).

Measuring light-induced transient absorption changes at 325 nm, reflecting the turnover of the primary quinone acceptor Q_A. Results are likewise summarized in Table 1.

Reverse phase HPLC analysis of the pigment content of monomeric and dimeric PSII complexes gave ratios of 16–19 Chl*a*/Pheo*a* and 0.8–2 PQ9/Pheo*a*. The mean values for monomeric, dimeric PSIIcc and redissolved crystals were 35, 34 and 34 Chl*a*/2 Pheo*a* and 2.3, 2.9 and 2.2 PQ9/36 Chl*a* (Table 2), respectively, indicating no significant differences between all three types of samples. A typical chromatogram for monomeric PSIIcc is shown in Fig 3c. As slight loss of Chl*a* by conversion to Pheo*a* during reverse phase chromatography could lead to an underestimation of the Chl*a* content, we used the maximum number of 36 Chl*a* found in the X-ray structures for calculating the composition of the other cofactors (see Table 2).

From the UV–VIS spectra of the acetone extract, the carotenoid/Chl*a* ratio of monomeric and dimeric PSIIcc was determined to be 0.231±0.03 and 0.253±0.03 Car/Chl*a*, yielding a total of 8.3±1 and 9.1±1 Car per 36 Chl*a*, respectively (Table 2). The slight difference in the Car content between monomeric and dimeric PSIIcc was also visible in the UV–VIS spectra (see Fig. 2a). Redissolved crystals showed the same Car content as dimeric PSIIcc prior to crystallisation (Table 2).

For examining the size of the detergent shell surrounding the protein complex, the detergent and lipid content of the solubilized monomeric and dimeric PSIIcc were determined performing TLC and GC analyses. Results of detergent and fatty acid quantifications assuming 36 Chl*a*/P680 are shown in Table 2. The obtained ratios were 3 βDM/Chl*a* and 0.56 fatty acids/Chl*a* for dimeric and 4 βDM/Chl*a* and 1.0 fatty acids/Chl*a* for monomeric PSIIcc.

The Mn-content of monomeric and dimeric PSII was analyzed by atom absorption spectroscopy. About 3.8 and 3.7 Mn/36 Chl*a* were found for dimeric and monomeric PSIIcc, respectively (Table 2). These results suggest that >95% of the oxygen evolving centres were functionally intact, if we take the measurements of light induced oxygen evolution activity (see above) into account.

Table 1
Oxygen evolution activities and Chl*a*/Q_A ratios for different PSII samples

Sample	Oxygen evolution activity		Chl*a*/Q_A
	Single flash Chl*a*(1/4 O_2 flash)$^{-1}$	Continuous saturating light μmol O_2 (mg Chl*a* h)$^{-1}$	
Crude extract	250–450	200–450	–
Monomeric PSIIcc	38–69	1700–3000	40–58
Dimeric PSIIcc	37–73	2200–3700	37–55
Redissolved crystals	41–55	2000–2600	39–51

Values are ranges obtained from at least six independent measurements for each sample type.

Table 2
Cofactor and lipid stoichiometry of monomeric and dimeric PSIIcc and redissolved crystals

	Monomeric PSIIcc	Dimeric PSIIcc	Redissolved crystals
Chl*a*[a]	35±2.6 (13)	34±2.1 (8)	34 ±2.2 (3)
Car[b]	8.3±1 (5)	9.1±1 (10)	9.1±0.8 (5)
PQ9[a]	2.3±1 (7)	2.9±0.8 (6)	2.2±0.2 (3)
Mn[b]	3.7±0.5 (3)	3.8±0.5 (4)	3.6±0.5 (4)
Lipids[b]	18±6 (5)	10±4 (6)	nd
βDM[b]	145±30 (6)	110±20 (6)	nd

Numbers in parentheses indicate number of independent experiments;nd: not determined due to high amount of protein required for measurement.
[a]values are per 2 Pheo*a*;
[b]values are per 36 Chl*a*;

3.3. Crystallisation and X-ray diffraction analysis

Using the micro-batch method, crystals of dimeric PSIIcc were obtained within 2 to 3 days. Crystal growth stalled after about 5 days. Crystals could be stored in the mother liquor for at least 2 to 3 weeks without visible deterioration either at RT or 4 °C, although sometimes extended storage leads to secondary nucleation and growth of small crystals on the surface of previously formed crystals. Crystals used for X-ray diffraction studies were 0.3 to 0.7 mm in the longest dimension and about 0.1 and 0.02 mm in the two other dimensions (Fig. 4a). They belong to the orthorhombic space group $P2_12_12_1$ with unit cell constants a=127.5, b=224.6, c=305.6 Å, as described previously [8,12]. Under cryogenic conditions the diffraction limit of these crystals using synchrotron X-ray radiation is 2.9 Å, as shown in Fig. 4b. Recently, a complete data set with a final resolution of 3.2 Å was collected at the ESRF beamline ID14-2 [7].

4. Discussion

The here described method allows rapid purification of large quantities of monomeric and dimeric PSIIcc from the thermophilic cyanobacterium *T. elongatus*. Using a novel photo bioreactor high cell densities could be obtained during cultivation, resulting in a yield of about 4 g cells per litre, compared to 1–1.5 g/l obtained with the previously used air lift fermenter [12,18]. Based on the former preparation protocol [11,12], the amount of protein purified per column run was increased by changing column and chromatography parameters. In order to avoid extended storage of protein between purification steps, the protocol was scaled up and optimised by shortening or skipping of time consuming steps. By using only βDM throughout the whole purification procedure, the formation of uniform micelles of known composition was ensured providing a homogeneous starting material for crystallisation. Subsequent detergent exchange steps were also avoided.

To separate monomeric and dimeric PSIIcc chromatographically, the flow rate and column geometry were crucial. The linear salt gradient used is very flat, i.e., the difference in salt ($MgSO_4$) concentration for eluting of monomeric and dimeric PSII is only 8 mM. The interaction between protein and column material (ToyoPearl DEAE 650 S) seems not to be of pure ionic nature, but is rather a mixture of hydrophobic interaction, size exclusion and anion exchange. This became obvious as monomeric PSIIcc sometimes detached from the column before the gradient was initiated, indicating that the interaction of monomeric PSIIcc with the column resin is not only dependent on the ionic strength of the eluent. In support of this observation, it should be noted that the matrix (HW55) of the applied column material is also used for size exclusion chromatography, although preferably in the molecular weight range below 100 kDa. Therefore, the choice of a rather long column with a small diameter for ion exchange chromatography was crucial for good separation of monomeric and dimeric PSIIcc.

With the described procedure, about 5% of Chl*a* of the crude cell extract were purified in form of monomeric and dimeric PSIIcc corresponding to ca. 200 mg PSII protein obtained from 100 g cells. A small amount (<5%) of dimeric

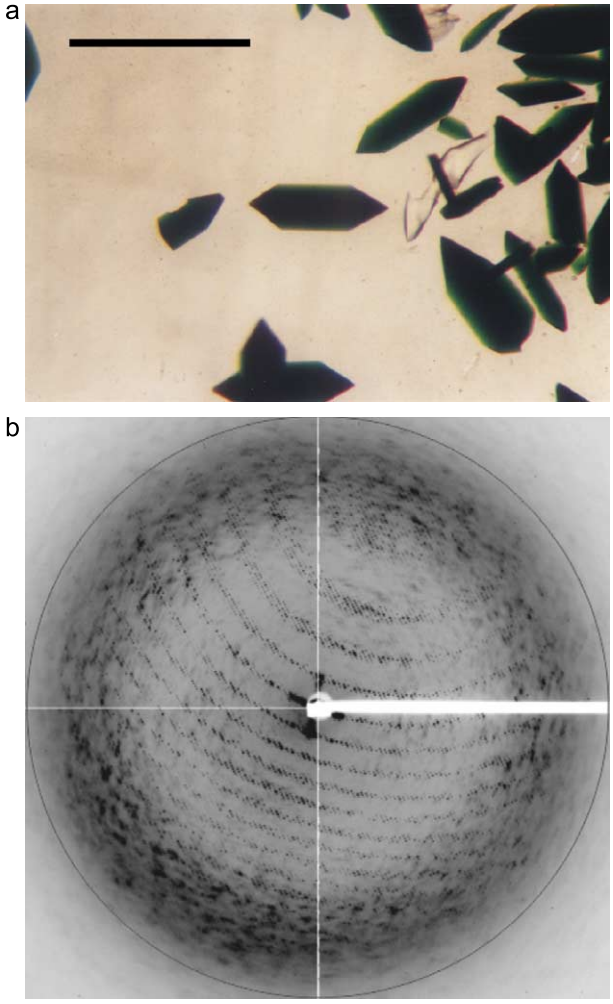

Fig. 4. (a) Crystals of PSIIcc from *T. elongatus*; bar=1 mm. (b) X-ray diffraction image of a PSIIcc crystal at 100 K, taken at beamline ID14-2 (wavelength 0.933 Å), ESRF, Grenoble; the ring indicates a resolution of 2.9 Å.

PSIIcc dissociated into monomeric PSIIcc during re-chromatography or storage at RT for several days (data not shown). Since this amount is too small to account for the total content of monomers, we conclude that the main fraction of monomeric PSIIcc is already present in the crude protein extract after solubilisation. Whether the dissociation of dimeric PSIIcc is induced by solubilisation with βDM or whether PSII is partly present in monomeric form in the thylakoid membranes is not yet clear. The variation of monomer to dimer ratio could either reflect a particular physiological state of the cells or be due to slight variations in solubilisation conditions within the different preparations (i.e., slight variations of protein/detergent and lipid/detergent ratio).

The size of the βDM solubilized PSIIcc complex in solution was determined by dynamic light scattering, size exclusion chromatography and analytical ultracentrifugation [34], supporting the assignment of the two obtained forms to monomeric and dimeric PSIIcc. In this study, the apparent molecular mass for monomeric PSIIcc from ultracentifugation experiments on diluted solutions (0.1 mg protein/ml) was 441±10 kDa, and for the dimeric PSIIcc a mass of 756±18 kDa was determined [34]. The subunit composition of the purified PSIIcc was examined in detail using SDS-PAGE, MALDI-TOF MS and N-terminal sequencing [33]. The presence of the following 19 subunits has been confirmed in monomeric and dimeric PSIIcc: PsbA, B, C, D, E, F, H, I, J, K, L, M, O, T, U, V, X, Y, Z (ycf9).

Using a different purification protocol, Kuhl et al. [22] likewise reported a small fraction of monomeric PSIIcc in their PSIIcc preparations from *T. elongatus*, but indicated that this monomeric PSIIcc is inactive in light induced oxygen evolution and did not contain any PsbO protein. By contrast, our monomeric PSIIcc showed the same subunit composition as the dimeric PSIIcc (see above and Ref. [33]), and both revealed no difference in activity. This is in agreement with an earlier study, reporting similar oxygen evolution activity (1/4 O_2/(80 Chl*a* flash)) for monomeric and dimeric PSIIcc form *T. elongatus* using sulfobetaine 12 as detergent and sucrose gradient centrifugation for separating both forms [13]. A mixture of monomeric and dimeric PSIIcc was also obtained from *T. elongatus* using Toyopearl DEAE 650 S and further separation by sucrose gradient centrifugation [19]. In contrast to our results, these authors found differences in both oxygen evolution activity and subunit composition of monomeric and dimeric PSIIcc.

Using the new type of photobioreactor in combination with the purification protocol described above, we obtained monomeric and dimeric PSIIcc with high oxygen evolution activity. The steady state oxygen evolution rates are comparable to values given in the literature for dimeric PSIIcc [22,35]. In addition, we measured oxygen evolving activities per single flash (see Table 1). Comparable data are missing in other studies. As various factors (e.g., double hits (two photons absorbed by the same photochemical centre) and misses (photons not absorbed by any photochemical centre)) are not accounted for in the measured number of centres, the real number of Chl*a* per oxygen evolving centre is lower. Assuming an error of about 10% due to these factors yields a corrected Chl*a* content of 35–40 Chl*a* per oxygen evolving centre in monomeric and dimeric PSIIcc. The slightly higher number of Chl*a* per photoreducible Q_A (37 to 58 in both preparations), determined by transient absorption changes at 325 nm, might be due to a small error in the difference extinction coefficient used [27]. Redissolved crystals showed the same oxygen evolution activity as dimeric PSIIcc prior to crystallisation (Table 1), and direct measurement of light-induced oxygen evolution from microcrystals showed an increased long-term stability when compared to that of PSIIcc in solution [36].

On the basis of 4 Mn/cluster and 36 Chl*a* per P680, the determined manganese content of 3.7 and 3.8 Mn/36 Chl*a* in monomeric and dimeric PSIIcc indicates that 93–95% of the centres contain a fully assembled Mn cluster. Similar Mn/Chl*a* stoichiometries (3.6 Mn/36 Chl*a*) were found when analysing redissolved crystals of dimeric PSIIcc, indicating that 90% of the water oxidizing complexes in the crystals contain an intact cluster. In addition to the data given, we measured the content of free Mn^{2+} (as hexaaquo complex in solution) in monomeric PSIIcc by electron paramagnetic resonance (EPR) at 9.4 GHz and RT (data not shown). For monomeric PSIIcc, usually less than 0.1 Mn/36 Chl*a* was found (the highest contamination found was about 0.36 Mn/36 Chl*a*). After treatment with 0.1 M NH_2OH, the same samples showed an increase in Mn^{2+} content of 3.4 to 3.7 Mn/36 Chl*a*. These data support the conclusion that >90% of the centres contain a fully assembled Mn cluster.

The Chl*a*/Pheo*a* stoichiometry of 17.5±1.2 is in good agreement with the 35 to 36 Chl*a* found per PSII centre in the X-ray structures [6–9] and reported for other PSII preparations from mesophilic and thermophilic cyanobacteria [22,37]. The determined number of 9±1 Car per PSII centre is in the range reported previously [17,37] (but see Ref. [38]), and redissolved crystals of dimeric PSIIcc showed within the experimental error the same Car/Chl*a* ratio. These findings are not in contrast to the fact that so far we can only localise one well-defined Car molecule in the electron density [7]. Due to the flexible structure of Car, allowing it to adopt a variety of different configurations, and the limited resolution of the X-ray diffraction patterns of the crystals, an assignment of electron density to Car molecules is difficult. Currently, we are not able to unambiguously assign more Car molecules, since they could easily be mixed up with phytol chains from Chl*a* molecules or with acyl chains from lipids. Reverse phase HPLC chromatograms always show a shoulder in the carotenoid peak which contributes to about 10% of the area of the main peak (Fig. 3c). This could be due to a modified β-carotene. Whether this other carotenoid species is already present in the PSIIcc or is artificially formed from β-carotene during the analysis is not yet clear.

Our chromatographic analysis shows that more than one PQ9 (about 2.9±0.8) is present per PSIIcc, as reported for

other PSIIcc preparations [17,37]. However, in the electron density calculated from the X-ray diffraction of our crystals (at 3.8, 3.6 and 3.2 Å) no PQ9 could be detected in the Q_B pocket [7,8,10]. Therefore, we conclude that the Q_B site is not fully occupied in our PSIIcc preparation. This is in line with flash-induced fluorescence measurements on solutions of our monomeric and dimeric PSIIcc, revealing that only 30% of monomeric and 50% of dimeric PSIIcc have a functional PQ9 in the Q_B site and that a small plastoquinone pool is present in both monomeric and dimeric PSIIcc (I. Vass, personal communication). This means that the chromatographically detected PQ9 molecules are partly located in the Q_A and Q_B sites and partly in the detergent shell or bound to the complex in a different, functionally inactive position. Ohno et al. [17] found 2.1 PQ9 per PSII from *T. elongatus* by chromatography. Monitoring $Q_A^{-\cdot}$ reoxidation in the absence and presence of DCMU, a Q_B content between 0% and 50% of the reduced Q_A was found [17] in accordance with our value. Similarly, 2.1 PQ9 per centre was determined in a highly purified PSII complex from *Synechocystis* PCC6803 [37], but only in a small fraction of centres could $Q_A^{-\cdot}$ be reoxidized by Q_B in the millisecond time range. Consequently, it was concluded that the second PQ9 molecule is not located in the Q_B site. These data as well as our own are in contradiction to the data reported for a PSIIcc preparation from *T. elongatus* by Kuhl et al. [22]. They infer from thermoluminescence measurements that their preparations have nearly 100% occupied Q_B sites with PQ9, but no experimental proof for the assignment of the thermoluminescence band using herbicides is given in this publication.

The determined size of the detergent shell (about 170 and 240 molecules (βDM and lipid)) per monomeric and dimeric PSIIcc, respectively, is in agreement with that calculated from titration experiments on detergent depleted PSIIcc. These studies have shown that 190±10 and 360±30 βDM molecules are necessary for redissolving detergent depleted monomeric and dimeric PSIIcc, respectively (F. Müh and A. Zouni, personal communication). From the structural model at 3.2 Å resolution [7] the membrane-embedded surface area for monomeric and dimeric PSIIcc can be estimated to be 12,600 and 20,700 $Å^2$, respectively. If we take the determined number of molecules in the detergent shell into account, a mean area of 65–90 $Å^2$ per detergent or lipid molecule can be calculated. This value is in the same order of magnitude as that from published data for the area per lipid molecule in monolayers (50–60 $Å^2$ [39,40]). We suggest that 20 lipids per detergent shell is rather the lower limit since lipid loss during quantification is likely. Ohno et al. determined about 60 lipids per P680 of PSIIcc from *T. elongatus*, using a different purification protocol with *n*-octyl glucopyranoside as detergent [17]. Presently, we cannot distinguish lipid molecules bound to the protein from those integrated into the detergent shell. Nevertheless, it seems likely that some of the lipid molecules will have a structural role in the PSIIcc in line with several studies indicating lipid influences on the assembly and function of PSII [41–47]. Likewise in other membrane protein complexes structurally and functionally important lipids were found. For example, in the crystal structure of PSI from *T. elongatus* one lipid was identified coordinating one of the antenna Chl*a* [48], and in the purple bacterial reaction centre two out of three lipids were localised close to the pigments of the electron transfer chain, maybe modulating their properties [49,50].

5. Conclusions and outlook

The cultivation of *T. elongatus* was optimised using a new type of photobioreactor. A fast and mild purification protocol for PSIIcc was developed avoiding high salt concentrations, strongly interacting column material or sucrose gradient centrifugation. For the first time, we obtained both homogeneous dimeric and monomeric PSIIcc of roughly the same subunit and pigment composition, both showing high oxygen evolving activity.

This purified dimeric PSIIcc from *T. elongatus* proved to be very stable providing the basis for improving the crystal quality. The crystals diffracted X-ray radiation to a maximum resolution of 2.9 Å at cryogenic temperatures using synchrotron facilities. The data sets collected from these crystals were processed to a resolution of 3.2 Å, and a detailed structural model was obtained [7]. This complements earlier and recent structural models for thermophilic cyanobacterial PSII at 3.8, 3.7 and 3.5 Å resolution, respectively [6,8,9].

Hitherto all crystals reported for cyanobacterial PSIIcc [6,12,22,35] are grown under comparable conditions (medium size PEG as precipitant) and they exhibit the same orthorhombic space group, cell constants and approximately the same X-ray diffraction properties. The currently achieved resolution does not allow to elucidate various details under debate in the structure. Getting a structural model at the resolution needed to clarify these details (<2.8 Å), better diffracting crystals are a prerequisite, maybe grown under different crystallisation conditions than applied until now. Searching for suitable conditions necessitates large amounts of homogeneous protein. The here described purification protocol fulfills these premises and is therefore a first step to reach this goal.

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