THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 27, Issue of July 7, pp. 20652–20659, 2000

Printed in U.S.A.

Towards Structural Determination of the Water-splitting Enzyme

PURIFICATION, CRYSTALLIZATION, AND PRELIMINARY CRYSTALLOGRAPHIC STUDIES OF PHOTOSYSTEM II FROM A THERMOPHILIC CYANOBACTERIUM*

Received for publication, February 16, 2000, and in revised form, March 29, 2000
Published, JBC Papers in Press, March 30, 2000, DOI 10.1074/jbc.M001321200

Helena Kuhl‡, Jochen Kruip‡, Andreas Seidler‡, Anja Krieger-Liszkay§, Marion Bünker‡, Dirk Bald¶, Axel J. Scheidig‖, and Matthias Rögner‡**

From the Department of ‡Plant Biochemistry, Faculty of Biology, Ruhr-University Bochum, D-44780 Bochum, Germany; the Department of §Plant Biochemistry, Institute of Biology II, University Freiburg, D-79104 Freiburg, Germany; ¶CREST Team 13, Tokyo Institute of Technology, Yokohama 226-8503, Japan; and the ‖Max-Planck-Institute for Molecular Physiology, D-44227 Dortmund, Germany

A photosystem II preparation from the thermophilic cyanobacterium *Synechococcus elongatus*, which is especially suitable for three-dimensional crystallization in a fully active form was developed. The efficient purification method applied here yielded 10 mg of protein of a homogenous dimeric complex of about 500 kDa within 2 days. Detailed characterization of the preparation demonstrated a fully active electron transport chain from the manganese cluster to plastoquinone in the Q_B binding site. The oxygen-evolving activity, 5000–6000 μmol of O_2/(h·mg of chlorophyll), was the highest so far reported and is maintained even at temperatures as high as 50 °C. The crystals obtained by the vapor diffusion method diffracted to a resolution of 4.3 Å. The space group was determined to be $P2_12_12_1$ with four photosystem II dimers per unit cell. Analysis of the redissolved crystals revealed that activity, supramolecular organization, and subunit composition were maintained during crystallization.

Photosystem II (PS II)[1] functions as a water/plastoquinone oxidoreductase in the thylakoid membrane of chloroplasts and cyanobacteria yielding oxygen, protons, and reduced plastoquinone by the splitting of water. Powered by sunlight, this is the key reaction that created and still sustains an oxygenic atmosphere on our planet. As a pigment-protein complex, PS II consists of more than 20 different subunits (1), most of them being integral membrane proteins. The PS II reaction center (RC) itself is composed of the D1 and D2 proteins, which bind the cofactors for the light-driven electron transfer processes (2). Upon light absorption, an electron is transferred from the primary electron donor P680 to a nearby pheophytin molecule. This charge separation is stabilized by rapid electron transfer to a tightly bound plastoquinone molecule, Q_A, and finally a mobile plastoquinone, Q_B, on the stromal/cytoplasmic side of the membrane. After double reduction and protonation, the reduced Q_B is released from its binding site on the D1 protein and replaced by an oxidized plastoquinone molecule. On the donor side, $P680^+$ is reduced by a tyrosine residue of the D1 protein, tyrZ (3–5), which in turn is reduced by a cluster of four manganese atoms located on the luminal side of PS II. This manganese cluster is the place where water splitting occurs. In four successive charge separation steps, four positive charge equivalents are accumulated that yield, finally, one molecule of oxygen out of two molecules of water. Accordingly, the manganese cluster can assume five different oxidation states, S_0–S_4, with S_4 being the state in which oxygen is released. Because water is a very stable molecule, its oxidation requires a potential of +1 V. Therefore, PS II is the most oxidizing enzyme in nature.

Closely associated with the reaction center is cytochrome b_{559} consisting of two subunits, α and β. Each subunit of the cytochrome provides one histidine side chain for heme binding. The function of cytochrome b_{559} is still not completely understood (for a recent review see Ref. 6). Two chlorophyll a (Chl a) binding proteins, CP47 and CP43, which form the inner antenna of the photosystem, are located on either side of the D1/D2 RC (7). Both proteins bind about 14–16 Chl a molecules (7, 8, 15) as well as three to five carotenoids. There is also a number of low molecular mass subunits (less than 10 kDa) attached to the PS II core complex, which are predicted to have only one transmembrane helix (9). Some of them seem to be dispensable for PS II function and assembly, whereas others are not. However, the exact function of the latter remains to be elucidated.

The manganese cluster at the luminal side of PS II is shielded by three extrinsic subunits (see Ref. 10 for a recent review). Higher plant PS II contains three subunits of 33, 23, and 16 kDa. Their role is to stabilize and to protect the manganese cluster as well as to increase binding of Ca^{2+} and Cl^-, two other inorganic cofactors of water splitting. In cyanobacteria only the 33-kDa protein exists. The 23- and 16-kDa proteins are replaced by cytochrome c_{550} and a 12-kDa protein, which seem to also have protective and stabilizing functions for PS II.

The relative positioning of the extrinsic subunits in the PS II complex was possible by comparison of the structure of PS II with and without these subunits by single particle analysis. This allowed the assignment of electron densities for the 33-kDa protein (11), the 23- and 16-kDa proteins in higher plant PS II (12), as well as the cytochrome c_{550} and the 12-Da protein in cyanobacterial PS II (13).

* The support by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 480, project C1 (to M. R.) and grant (to H. K.) from Graduiertenkolleg) is gratefully acknowledged. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Plant Biochemistry, Faculty of Biology, Ruhr-University Bochum, Universitätsstrasse 150, D-44780 Bochum, Germany. Tel.: 49-234-3223634; Fax: 49-234-3214322; E-mail: matthias.roegner@ruhr-uni-bochum.de.

[1] The abbreviations used are: PS II, photosystem II; MES, 2-(*N*-morpholino)ethanesulfonic acid; β-DM, β-dodecyl-D-maltoside; Chl, chlorophyll; RC, reaction center; HPLC, high performance liquid chromatography; HIC, hydrophobic interaction chromatography; PAGE, polyacrylamide gel electrophoresis; 2,6-DCBQ, 2,6-dichloro-*p*-benzoquinone.

Despite intense research over the last two decades, the nature and function of the key components of PS II, the primary electron donor P680 which generates the oxidative power to split water and the manganese cluster where water splitting takes place, are still poorly understood. Although there has been considerable progress in the structural analysis of PS II in recent years, knowledge about its high resolution structure is still missing. The first detailed three-dimensional structure of PS II at 8-Å resolution was obtained by cryoelectron microscopy of two-dimensional crystals obtained from a so-called RC·CP47 complex (14, 15). This complex consists of the D1 and D2 reaction center proteins as well as cytochrome b_{559}, CP47, and probably some of the small hydrophobic subunits. The electron density of 23 transmembrane helices was assigned as well as a number of tetrapyrrole rings of chlorophylls, pheophytins, or heme groups. Comparison with the structure of the bacterial reaction center (16, 17) as well as with PS I (18) allowed the assignment of 10 helices to D1/D2 RC and six to CP47. In addition, a projection map of a PS II core complex retaining CP43 and the extrinsic 33-kDa protein was reported, which allowed the positioning of CP43 within PS II (7). However, the fact that most two- and three-dimensional crystallization attempts of PS II preparations from mesophilic organisms resulted in the loss of the luminal side subunits and/or the water-splitting capacity (see for example Refs. 14 and 19) points out the importance of using more stable PS II complexes for crystallization, for example those isolated from thermophilic cyanobacteria.

In this communication we present a new purification procedure for highly active dimeric PS II complexes with intact electron donor and acceptor sides. Three-dimensional crystals were obtained, which diffract to about 4.3-Å resolution. A first characterization of these crystals together with a detailed biochemical and biophysical analysis shows the extraordinary stability of these PS II complexes, which maintain both their dimeric state and high oxygen-evolving activity even during crystallization.

EXPERIMENTAL PROCEDURES

Cell Growth and Thylakoid Preparation—Synechococcus elongatus was grown in a 25-liter foil fermenter (Bioengineering, Switzerland) at 55 °C in special growth medium according to a previous study (20), bubbled with CO_2-enriched air (5%) and illuminated with white light of increasing intensity (50–250 μEinstein). Cells were harvested after 3–4 days at an OD_{730} of 3–4 and concentrated to about 1.5 liters using a hollow fiber system (Amicon DC10 LA). Pelleting by centrifugation (Sorvall GSA rotor, 11,000 rpm, 18 min, 4 °C) yielded about 100 g of cells (wet weight), which were resuspended in about 150 ml of buffer A (20 mM MES, pH 6.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$) and pelleted again. After resuspension in about 200 ml of buffer B (20 mM MES, pH 6.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5 M mannitol), lysozyme was added to a final concentration of 1 g/liter. Cells were incubated in the dark at 37–40 °C for 90 min on a shaker. Upon adding an aliquot of DNase and RNase, cells were disrupted using a Parr bomb, pelleted (Sorvall GSA rotor, 11,000 rpm, 18 min, 4 °C), and resuspended in 200 ml of buffer A. This washing step was repeated twice. The pellet was resuspended in 200 ml of buffer B, centrifuged again, and resuspended in a small volume of buffer B + 20% glycerol to yield a high Chl concentration. The membranes were frozen in liquid nitrogen and stored at −70 °C.

Purification of PS II Core Complexes by High Performance Liquid Chromatography (HPLC)—Thylakoid membranes were washed with 0.05% β-dodecyl-D-maltoside (β-DM) (Biomol), pelleted, and resuspended in extraction buffer (20 mM HEPES, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.2 M $(NH_4)_2SO_4$). Membrane proteins were solubilized at a chlorophyll concentration of 1 mg of Chl/ml with 1.2% β-DM (Biomol) and 0.5% sodium cholate (Sigma) by stirring in the dark at room temperature for 30 min. After centrifugation (Sorvall T647.5 rotor, 40,000 rpm, 1 h, 4 °C) AMS buffer (3 M $(NH_4)_2SO_4$, 20 mM HEPES, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$) was added to the supernatant to a final $(NH_4)_2SO_4$ concentration of 1.65 M. The extract was loaded onto a hydrophobic interaction chromatography (HIC) column (POROS ET, PE Biosystems) connected to a perfusion chromatography workstation (BioCAD 700E, PE Biosystems). The HIC column was previously equilibrated with 1.65 M $(NH_4)_2SO_4$, 20 mM HEPES, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.03% β-DM. PS II was eluted after a short wash step with a gradient of 1.65–0 M $(NH_4)_2SO_4$ in the same buffer at a flow rate of 10 ml/min. PS II-containing fractions were pooled and concentrated in a concentration device (Amicon Model 8050, equipped with a YM-100 membrane) to 8–10 ml followed by overnight dialysis against 20 mM MES, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, 0.03% β-DM. The sample was loaded onto an anion-exchange column (UNO Q-6, Bio-Rad) pre-equilibrated with the same buffer containing 5 mM $MgSO_4$ and eluted with a linear gradient of 5–200 mM $MgSO_4$ in the same buffer at a flow rate of 4 ml/min. Fractions containing active PS II were pooled and concentrated twice by ultrafiltration (cutoff 100 kDa) to finally 2–10 mg of Chl/ml. The sample was frozen in liquid nitrogen and stored at −70 °C.

Analytical Size Exclusion HPLC—Analytical size-exclusion chromatography was performed on a Waters system consisting of a photodiode array detector 996, pump 515, and an injector (Rheodyne) with a TSK-gel 4000 SW_{XL} column (TosoHaas, Stuttgart) using a buffer of 20 mM MES, pH 6.5, 30 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, and 0.03% β-dodecyl maltoside at a flow rate of 0.5 ml min^{-1}.

Pigment Analysis by Reversed Phase Chromatography—Purified PS II complexes were mixed with acetone to yield a final acetone concentration of 87%. After vortexing and centrifugation (Biofuge, 15 min, 13,000 rpm, 4 °C), the pelleted protein was separated from the supernatant containing the extracted pigments. Filtrated supernatant (up to 50 ng of Chl) was applied onto a Reversed phase column (Spherisorb Typ ODS1, particle size 5 μm), which had been pre-equilibrated with buffer A (acetonitrile, methanol, 0.1 M Tris-HCl (pH 8.0) mixed in a ratio of 72:8:3) at 20 °C. At a flow rate of 1.5 ml/min, a linear gradient up to 100% of buffer B (methanol and hexane at a ratio 7:1) was applied. Quantification of chlorophyll *a* relative to pheophytin was done according to the elution profile recorded at 663 nm, using the respective peak areas and extinction coefficients of 78.4 mM^{-1} cm^{-1} (Chl *a*) and 49.6 mM^{-1} cm^{-1} (Pheo) (according to Ref. 21, modified). All organic solvents used were of HPLC grade (Baker) and have been degassed prior to use.

Measurement of Oxygen Evolution—Rates of oxygen evolution were measured at 25 °C unless otherwise indicated in a thermostated Clark-type oxygen electrode (Model RE K1-1 N, Biolytik, Germany) using continuous, saturating light provided by a 250-W cold light source (Halolux). Thylakoid membranes, extract, and PS II core complexes were suspended in 20 mM MES, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, 0.03% β-DM with a concentration of 2–5 μg Chl/ml. As artificial electron acceptors 2,6-dichloro-*p*-benzoquinone (2,6-DCBQ) or ferricyanide were used, both with a final concentration of 2 mM.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)—The polypeptide composition of the core complexes was analyzed using denaturing SDS-PAGE with a 12–22% linear gradient polyacrylamide gel according to a modified Laemmli system with 0.75 M Tris in the separating gel. Samples were solubilized in sample buffer with 3% SDS and 5% β-mercaptoethanol (5 min at 60 °C and, subsequently, 45 min at room temperature). For heme staining, β-mercaptoethanol was omitted. The gels were stained with Coomassie Brilliant Blue R-250 or tetramethyl benzidine according to a previous study (22).

Optical Measurements—Absorption spectroscopy was performed on a Beckman DU-7400 spectrophotometer. Chlorophyll concentration was determined after extraction from thylakoid membranes with 100% methanol by its absorbance at 665 and 649 nm according to a previous study (21).

For optical difference spectra, the isolated PS II complex (10 μg of Chl/ml) was treated with ferricyanide (final concentration, 2 mM) to oxidize the heme groups, followed by partial reduction by ascorbate (final concentration, 10 mM) and full reduction by the addition of an aliquot of dithionite. At each stage, 10 spectra were recorded and averaged prior to subtraction.

Thermoluminescence Measurements—Thermoluminescence was recorded in a set-up described previously (23) using PS II samples at a Chl concentration of 17 μg of Chl/ml. Thermoluminescence was charged at −15 °C or 3 °C, respectively, by applying one or more single turnover flashes onto the samples. Signals were recorded during warming up the sample to 70 °C at a heating rate of 20 °C/min. Prior to thermoluminescence measurements, samples were dark-adapted for 2 min at 20 °C. No cryoprotectants were added for the measurements. Similar thermoluminescence bands were obtained with two different preparations of isolated PS II complexes.

Crystallization Procedure—Best crystals were obtained with a reservoir consisting of 10% (w/v) polyethylene glycol 4000, 100 mM HEPES-NaOH (pH 7.5), and 100 mM ammonium sulfate. Different techniques

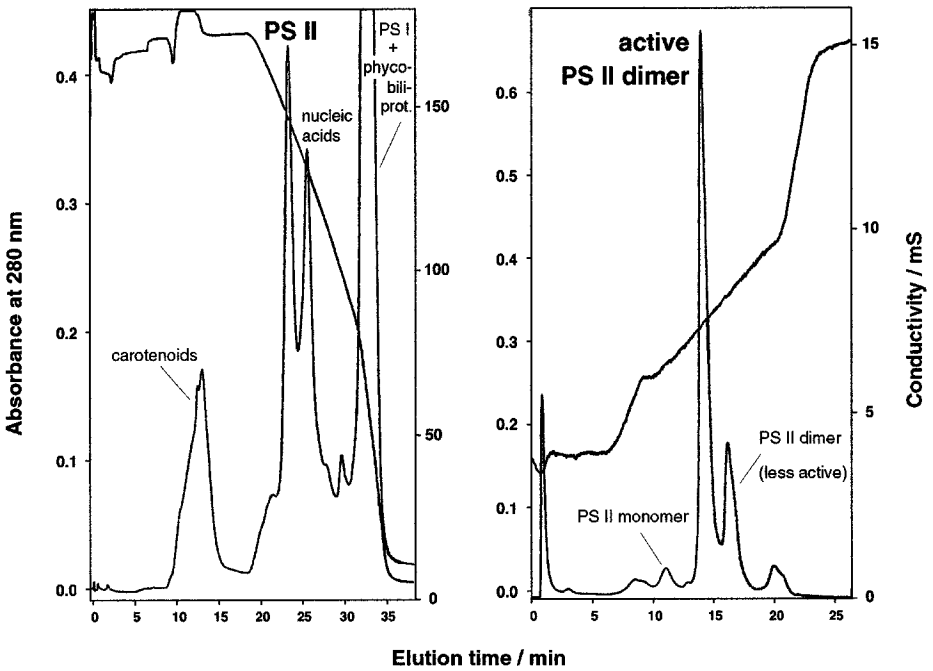

FIG. 1. **Elution profiles from the first and second chromatographic step, HIC, and ion-exchange chromatography column.** The flow rates for the two column runs were 10 and 4 ml/min, respectively (for details see "Experimental Procedures").

for the crystallization were successfully applied: Vapor diffusion in Linbro cell culture plates using "hanging drop" and "sitting drop" setups equilibrating against a 1.0-ml reservoir (24), micro batch under oil (25), micro batch in small 400-μl chambers, and micro batch in crystallization capillaries. In each case, the ratios of protein solution to reservoir solution were between 1:1 and 2:1, respectively. The final drop volume was between 1.0 and 3.0 μl. In all cases the glass surfaces in contact with the protein solution were siliconized with a solution of 0.5% (v/v) dimethyl dichlorosilane in toluene.

RESULTS

Isolation and Biochemical Characterization of a Dimeric PS II Complex—Important requirements for the successful crystallization of a multisubunit protein complex with many cofactors such as PS II are the stability, homogeneity, intactness, and sufficient quantities of the protein complex. For cyanobacterial PS II, this involves an efficient mass culture (see "Experimental Procedures"), an extraction procedure using mild detergents without loss of activity or partial loss of (extrinsic) subunits, and a fast and efficient purification procedure. Although other details are outlined in the "Experimental Procedures," the extraction and the chromatographic purification procedures, which are the key steps, should be evaluated separately as follows:

(*a*) Extraction of PS II from the crude thylakoid membranes was optimized using a combination of the detergents β-dodecyl-D-maltoside and sodium cholate. Although the addition of sodium cholate led to a reduced solubilization efficiency compared with solubilization by β-DM alone, monomerization of PS II was avoided, which is an important prerequisite for a homogenous preparation and high oxygen activity yields.

(*b*) The use of perfusion chromatography as the first chromatographic step resulted in a considerable acceleration of the purification procedure: Due to the stability of the matrix material with excellent flow characteristics, the crude extract could be applied directly onto the column without any prepurification step, and the flow rates could be increased up to a factor of 10. Using ethyl surface groups, weak hydrophobic interaction chromatography could separate PS II from the other main protein components of the thylakoid membrane, *i.e.* PS I (absorption maximum at 680 nm) (Fig. 1, *left*) and phycobiliproteins (absorption maximum at 600–650 nm). In addition, free carotenoids (first peak of the elution diagram) and nucleic acids (peak next to PS II) were separated, as identified by absorption spectroscopy. In a second step, an ion-exchange column with continuous bed matrix material separated PS II dimers with high and low oxygen-evolving activity (Fig. 1, *right*). The reason for the lower activity of one fraction (only 30–50% of the maximum activity) is not yet clear, because SDS-PAGE analysis did not show any significant difference between the two peaks. The dimeric state of both PS II fractions was verified by size exclusion HPLC (see also Fig. 6, *left*). Routinely, also a small peak of inactive monomers lacking the 33-kDa subunit (verified by size exclusion HPLC and SDS-PAGE analysis, data not shown) was observed. In conclusion, this second step was decisive in achieving both structural and functional homogeneity of the dimeric PS II complex. Also, besides using new column technology, the use of the mild detergent β-DM for all steps, including solubilization of the thylakoid membrane, was a prerequisite to maintaining a very high oxygen evolution activity of the isolated complexes.

The success of this PS II purification procedure was analyzed by SDS-PAGE and absorption spectroscopy (Fig. 2). By SDS-PAGE analysis of samples taken at various steps during purification, from thylakoid membranes (Fig. 2, *left*, *lane b*) to purified PS II (*lane e*), the extreme efficiency of the HIC step, and the further removal of numerous contaminating proteins by the ion-exchange chromatography step is obvious. Especially, the loss of phycobilisome subunits (17–20 kDa) by the HIC step is apparent (*lane d*). Protein subunits of the purified PS II complex (*lane e*) were assigned according to their apparent molecular masses and/or Western blotting (data not shown). All major PS II subunits are present: The reaction center proteins D1 and D2, the internal antenna proteins CP47 and CP43; the α-subunit of cytochrome b_{559} (PsbE); and the three lumen-exposed subunits, the 33-kDa protein, cytochrome c_{550}, and the 12-kDa protein, which are decisive for preserving the water-splitting activity. Additionally, at least three small subunits with molecular masses between 2 and 8 kDa were detected, including most probably the β-subunit of cytochrome b_{559}. The presence of cytochrome c_{550} and the 12-kDa protein was previously confirmed by immunoblot analysis and heme staining (13).

The success of the purification procedure is also obvious

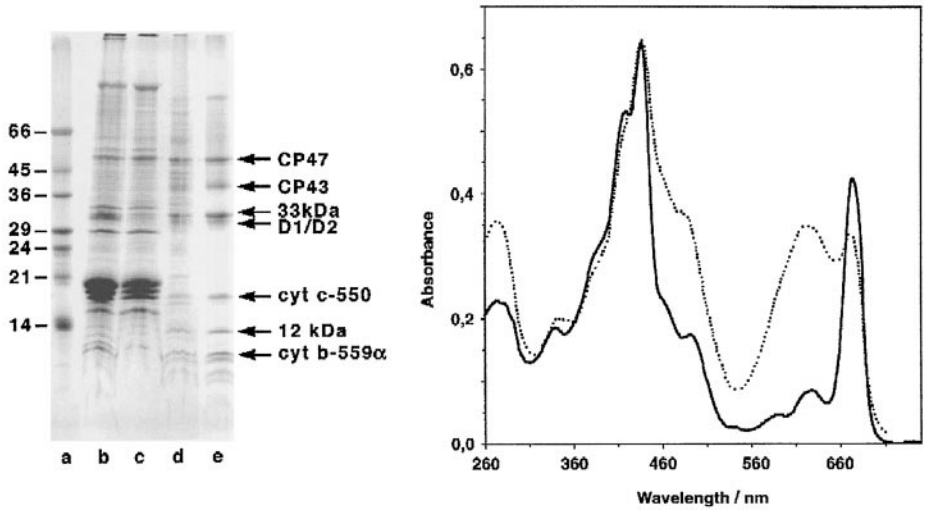

FIG. 2. **SDS-PAGE (*A*) and absorption spectra (*B*) taken at various steps during the isolation procedure of PS II.** *Left*, SDS-PAGE showing molecular mass markers (*a*), thylakoid membranes (*b*), solubilized membrane proteins (*c*), and PS II after the first (*d*) and second (*e*) HPLC purification steps. *Right*, absorption spectra of PS II after membrane extraction (*dotted curve*) and PS II purified by two HPLC steps (*solid line*).

when comparing absorption spectra of the crude extract and the isolated PS II complex (Fig. 2, *right*). In contrast to the extract with prevailing PS I (peaking at 679 nm) and phycobilisomes (600–650 nm), the purified PS II core complex shows the characteristic absorption maximum at about 674 nm and the loss of all contaminating chromophores.

This purity as analyzed by UV-visible spectroscopy and gel electrophoresis is in line with a low and reproducible number of chlorophylls per 2 pheophytin molecules as determined by reversed phase chromatography. Several determinations of various preparations yielded (39 ± 1) Chl per reaction center, based on 2 pheophytins per PS II core complex (data not shown). The small deviation from the average value indicates the excellent reproducibility of the preparation procedure.

Characterization of the Electron Donor/Water-splitting and Electron Acceptor Sides—The purified PS II dimers showed a very high oxygen-evolving activity with a typical rate of 5000–6000 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} (at 25 °C with 2,6-DCBQ as electron acceptor). To our knowledge, this is the highest rate reported for a PS II preparation. Also, the preparation showed a remarkable stability, retaining about 70–80% of its original activity after 7 days of storage on ice in the dark, which is an excellent basis for subsequent crystallization experiments.

In accordance with oxygen evolution measurements on intact cells (26), activity measurements with ferricyanide as electron acceptor yielded an optimum above 50 °C for isolated complexes, whereas the electron acceptor 2,6-DCBQ decomposes at temperatures higher than 35 °C. The high temperature optimum for the oxygen evolution activity indicates that the thermostability of the water-splitting complex was maintained during the purification procedure and is an inherent property of this protein complex.

To study the nature of the cytochromes present in the PS II preparation, redox difference spectra were recorded. The dithionite-reduced minus ascorbate-oxidized difference spectrum showed peaks at 550 and 522 nm (Fig. 3, *left*). These maxima are characteristic for cytochrome c_{550} (27, 28); the apparent low redox potential is in agreement with the −250 mV level reported for cytochrome c_{550} for other cyanobacteria (28, 29). An ascorbate-reduced minus ferricyanide-oxidized difference spectrum showed merely the characteristic peak of cytochrome b_{559} at approximately 559 nm (Fig. 3, *left*, *dotted line* (6)).

Thermoluminescence measurements were performed to probe the functionality of the Q_B binding site of the isolated PS II complex. After excitation by single turnover flashes given at −15 °C or 3 °C, respectively, a thermoluminescence B-band at approximately 24 °C was observed, which originated from charge recombination of the semiquinone Q_B^- with the S_2/S_3 state of the manganese cluster (30). Fig. 3 (*right*) shows a thermoluminescence signal after excitation with two single turnover flashes. With the intensity of a single turnover flash being too low to yield saturation, two flashes were required to achieve maximal signal size for this preparation. In addition to the B-band, a small Q-band due to charge recombination of Q_A^- with the S_2/S_3 state of the manganese cluster was observed at 4 °C (30). This band reflects centers with Q_B missing or with blocked electron transport from Q_A to Q_B. From the ratio of the two bands, a 90–95% occupation of the Q_B site by plastoquinone was estimated, which is probably an underestimation: on one hand, the intensity of the excitation flash was still not saturating; on the other, part of the centers may have become double reduced after the second flash, being single reduced after the first flash.

In summary, the PS II preparation presented here is the first one that yields highly purified PS II complexes with an intact Q_B site, occupied by a plastoquinone molecule. This is an important fact in that it starts from a homogenous preparation then yields homogenous crystals for x-ray analysis.

Three-dimensional Crystallization and Crystal Analysis—Crystals were grown by the hanging drop method using polyethylene glycol 4000 and $(NH_4)_2SO_4$ as precipitants.

Depending on the conditions, crystals of different shape and size grew within 6 h. Fig. 4 shows two different crystallization results obtained by various conditions. If the equilibration of the drop was too fast or the precipitant concentration too high the crystals had the shape of dendrites (Fig. 4*A*), which did not show any x-ray diffraction. Under optimal conditions PS II crystallized in well shaped individual crystals reaching maximum dimensions of around 1.0 x 0.5 x 0.2 mm (Fig. 4, *B* and *C*).

For the optimization of the crystallization conditions, different temperatures were tested (4, 15, 18, and 22 °C) yielding an optimum at 18 °C.

Using the vapor diffusion techniques, we observed that the crystals dissolved after 1 week, presumably due to a continuous increase in the detergent and the salt concentration in the drop. Crystals grown under oil kept their shape and diffraction quality for over 2 weeks and yielded a diffraction of around 10 Å at room temperature using an in-house rotating anode. However, as they were extremely sensitive to x-ray radiation, cryoconditions had to be developed for further data collections. The extreme fragility of the crystals and their sensitivity against variations in the composition of the mother liquor hampered the development of the cryoconditions considerably; finally, the

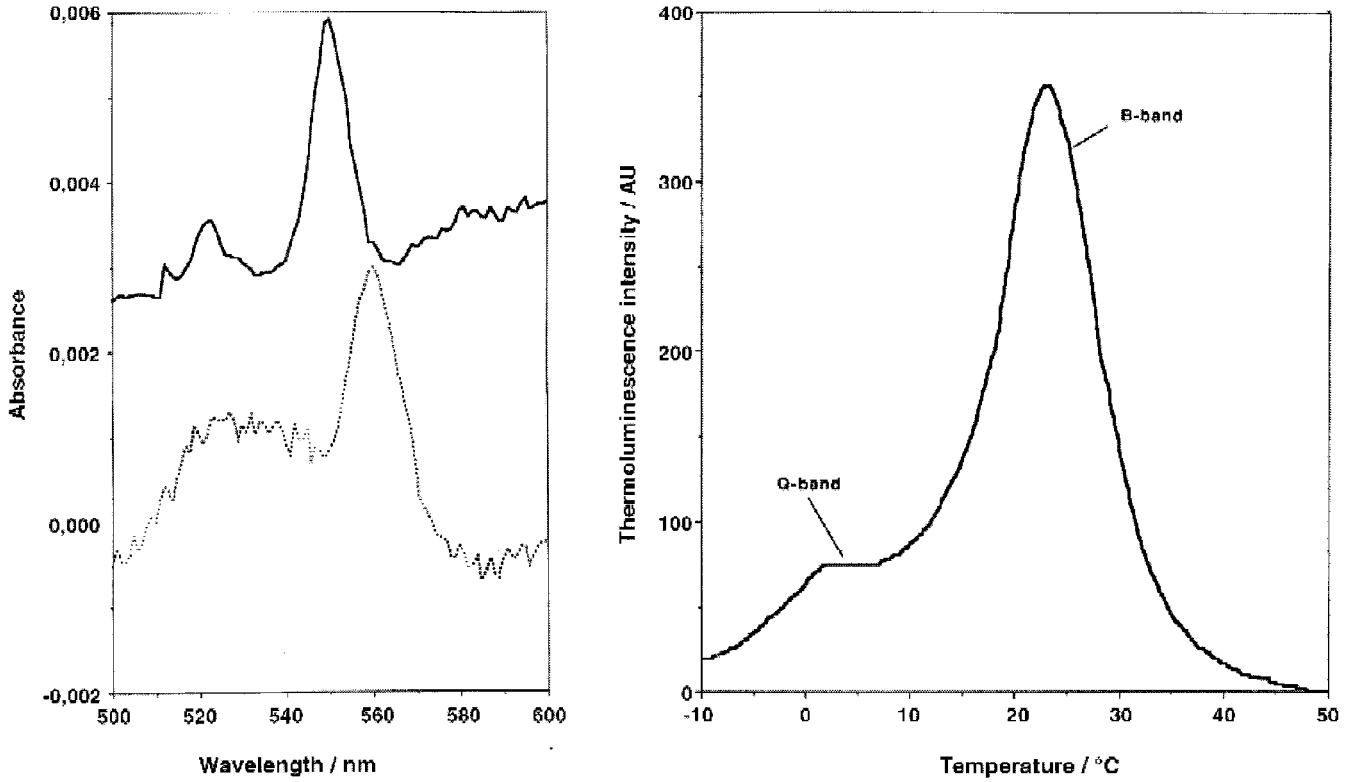

FIG. 3. *Left*, difference spectra of cytochrome c_{550} with dithionite-ascorbate (*solid line*) and cytochrome b_{559} with ascorbate-ferricyanide (*dotted line*). *Right*, thermoluminescence signal from purified PS II complexes. Thermoluminescence was excited by giving two flashes at 3 °C; the sample contained 14 μg/ml Chl.

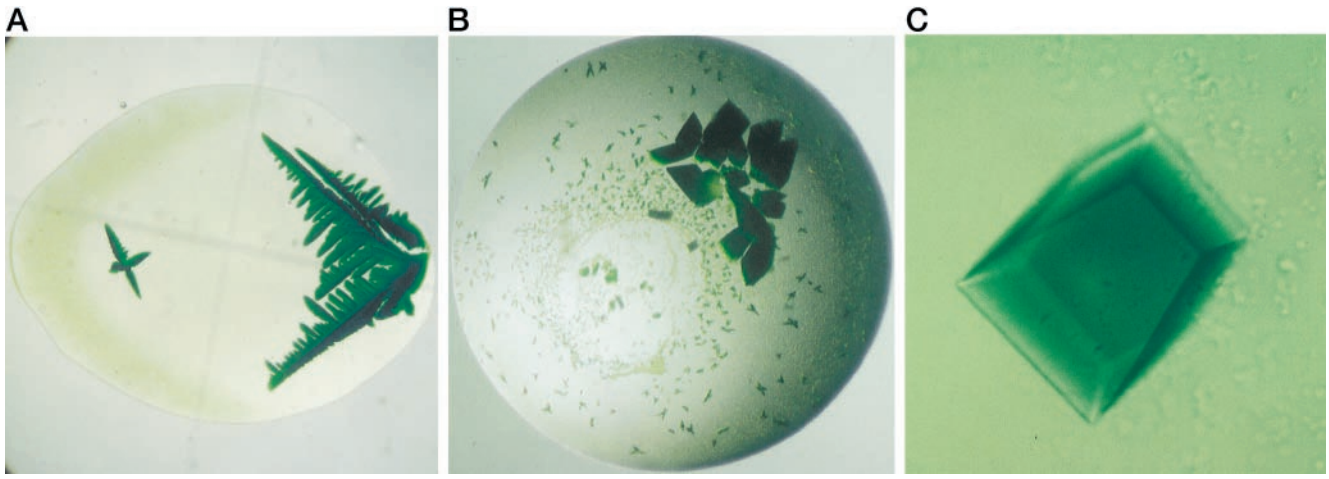

FIG. 4. **Light microscopic images of different three-dimensional PS II crystals.** *A*, a typical result of PS II crystals growing too fast (mostly the concentration of the precipitant was too high). Within 1 day large dendrites are formed, which display no x-ray diffraction at all. *B*, hanging drop under optimal conditions resulting in single crystals of PS II with dimensions of around $0.3 \times 0.3 \times 0.1$ mm. These crystals are visible within 2 h after mixing the drop and reach their final size after 2 days. In addition, after 1 week small dendrites are visible in the drop. *C*, enlarged view of a PS II single crystal.

best results were obtained by flash-freezing of small crystals in liquid nitrogen with glycerol as cryoprotectant.

The unit-cell dimensions and the space group were determined using a data set (99% complete to 7.5 Å) collected from a small single crystal ($<0.1 \times <0.1 \times < 0.05$ mm) at our in-house rotating copper anode (Enraf-Nonius FR 591) equipped with an x-ray mirror system (Supper Inc., Natick, MA) and an imaging plate detector (Mar345). The diffraction pattern revealed an orthorhombic space group, with the unit-cell parameters a = 139.6 Å, b = 225.8 Å, and c = 309.0 Å.

Under cryoconditions the PS II crystals diffracted in-house up to 7.5-Å resolution, however, they displayed large mosaicity, very diffuse scattering, and only 2 out of 10 crystals were single. In addition, we observed a significant decrease in crystal quality (in terms of mosaicity) with increasing size of the crystals.

To further evaluate the optimization of the crystallization- and cryoconditions, we used test beamtime at different synchrotron sources. The best data so far were obtained with very small crystals (largest dimension < 0.1 mm) at the microfocus beamline ID13 at the European Synchrotron Radiation Facility (Grenoble, France). A first preliminary data set with maximum resolution of around 4.3 Å could be collected (Fig. 5, *A* and *B*). However, the crystals displayed in the high flux beam a severe

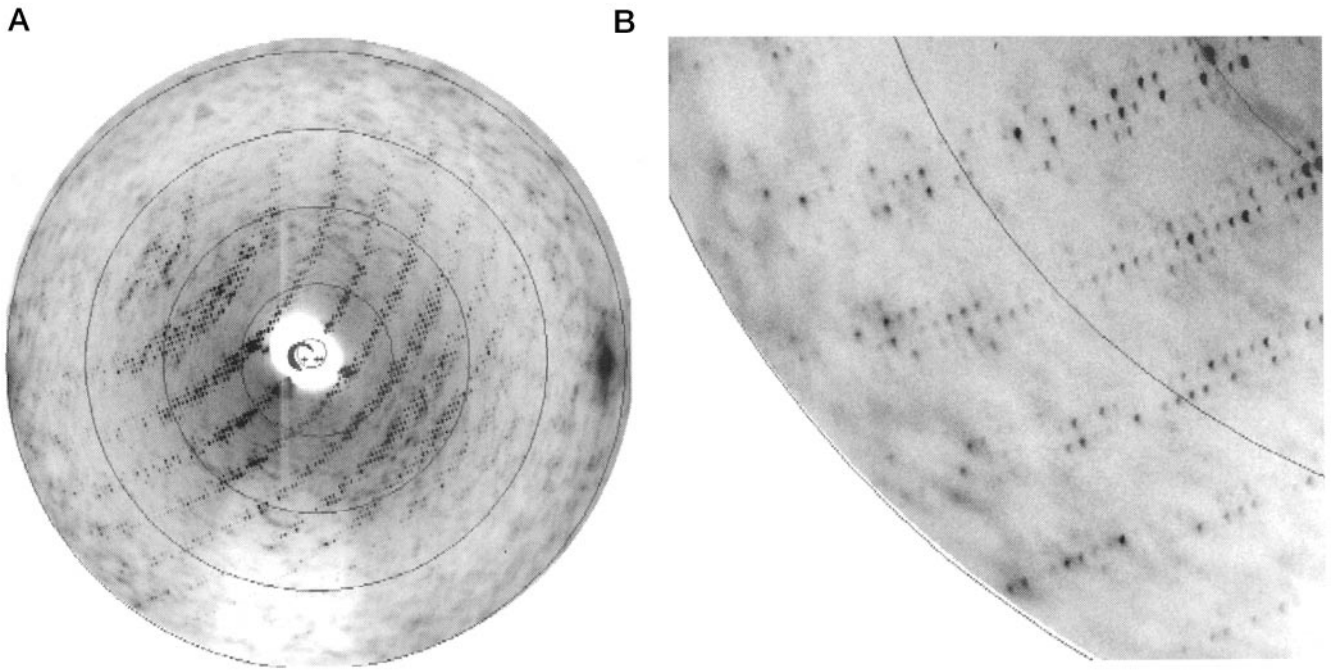

FIG. 5. **Oscillation x-ray diffraction pattern of a single PS II crystal.** *A*, diffraction pattern recorded from a crystal of PS II grown within 10 h at the European Synchrotron Radiation Facility and shock-frozen in liquid nitrogen. The experiment was performed at the microfocus beamline ID13 equipped with a MarCCD. Crystal-to-film distance, 350 mm; detector radius, 65 mm; wavelength, 0.782 Å; oscillation range, 1.0°; exposure time 30 s; temperature, 100 K. The edge of the detector is at 4.3-Å resolution. It can be seen that the diffraction is very anisotropic. *B*, enlarged section of the diffraction image displaying weak reflections up to the edge of the detector at a resolution of around 4.3 Å.

radiation damage even at 100 K, which did not enable us to collect a complete data set from a single crystal. The incompleteness of the collected data, the significant radiation damage, and the strong diffuse scattering contributed to a high merging *R*-factor (around 20%) for the obtained data set. Based on the systematic absences of reflections, we assumed the space group to be $P2_12_12_1$.

To proof structural and functional intactness of PS II within the crystal, we redissolved PS II crystals and compared their properties with the starting material of the crystallization assays. HPLC size exclusion chromatography revealed that PS II still was a dimeric complex after crystallization (Fig. 6, *left*). For comparison, monomeric PS II is also shown in these elution profiles. Additionally, the unchanged subunit composition is obvious from the SDS-PAGE analysis of the redissolved crystals in comparison with the pattern before crystallization (Fig. 6, *right*); namely, the extrinsic subunits, the 33-kDa protein, cytochrome c_{550}, and the 12-kDa protein, which are easily lost in PS II preparations of mesophilic organisms, were still present after crystallization. This structural integrity is consistent with the fact that the oxygen evolution activity is well preserved in the crystals: At least 70% of the original rate was measured with the redissolved crystals (data not shown).

Based on an averaged molecular mass of the PS II dimer in the detergent micelle of around 500 kDa, we estimated that the crystal contains one PS II dimer in the asymmetric unit with a Matthews coefficient (VM) of 4.7 $Å^3$/Da (31).

Cryo- and negative stain electron microscopy studies enabled us to estimate the dimensions of PS II dimers from the thermophilic cyanobacterium *S. elongatus* to be around 115 × 170 Å in the plane of the membrane and around 95 Å across the membrane (11, 13). Based on the electron microscopy pictures and the measured dimensions, we constructed a simplified model of a PS II dimer (Fig. 7, *A–C*) and placed it in the $P2_12_12_1$ cell with the observed cell dimensions. Taking the flexibility of the detergent ring into account, we filled the whole unit cell with four PS II dimers without overlap between neighbored dimers and without holes big enough to fit even a PS II monomer. The suggested model for the arrangement of PS II dimers is shown in Fig. 7 (*D–G*). We predict a packing between the lumen-exposed subunits and the cytoplasma-exposed site along the *c* axis. In this proposed packing, the surrounding detergent ring (membrane plane) would be in the *ab* plane of the unit cell.

DISCUSSION

The procedure for the isolation of PS II from *S. elongatus* described here is characterized by its simplicity and speed. In particular, due to the robust perfusion chromatography matrix material, the time-consuming overnight sucrose-density gradient required in the former procedure (32) can be omitted.

Besides PS II, the combination of two chromatographic steps involving porous matrix materials could be a useful strategy for the purification of membrane proteins in general; with slight modifications, this procedure already turned out to be extremely efficient for the purification of PS I (33, 34, 35) and the cytochrome b_6f complex (36) from various cyanobacteria. Especially, the hydrophobic interaction chromatography step achieves a powerful enrichment if the selection of the column material, in the case of membrane proteins with surface-exposed groups of very low hydrophobicity, is combined with optimal pH and salt conditions. Additionally, both chromatographic steps have also the potential for further scaling up due to the stability and excellent flow characteristics of these matrix materials**.**

In comparison with other PS II preparations from cyanobacteria or higher plants (for review see Ref. 37), our preparation contains all function relevant components of the electron donor and acceptor sides with highest degree of preservation. The isolated PS II shows the highest rate of oxygen evolution reported so far, which is in line with a clear multiline signal obtained from EPR measurements before, indicating a functional manganese cluster (38). High rates of oxygen evolution, up to 4500 μM O_2 per mg of Chl and per hour, have also been reported from another PS II preparation of the same organism

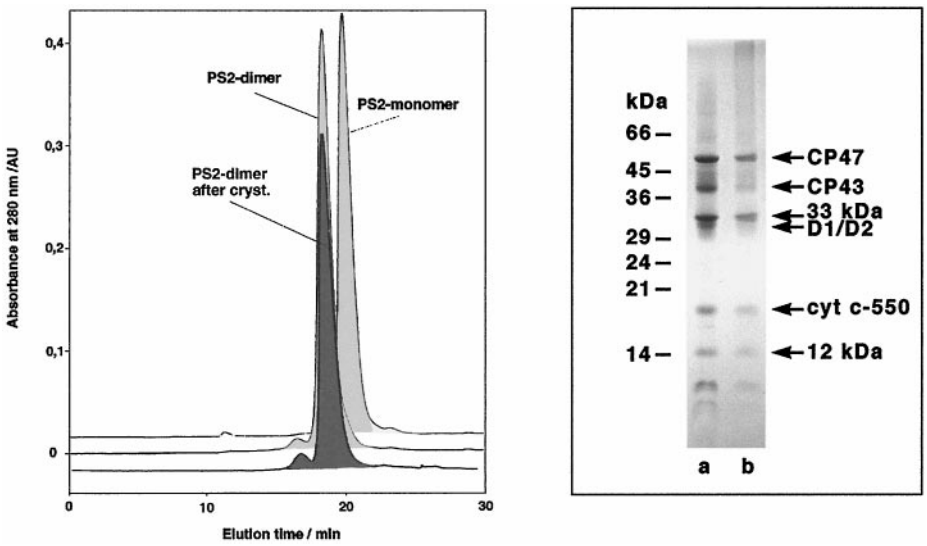

FIG. 6. **Comparison of size, homogeneity, and subunit composition of the dimeric PS II complex before and after crystallization.** 1–2 days after crystallization, crystals were resolubilized in buffer (20 mM MES, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, 0.03% β-DM) and characterized by: *Left*, size exclusion chromatography, using a TSKgel 4000 SW_{XL} (see "Experimental Procedures"); for comparison, also the elution profile of monomeric PS II is shown. *Right*, SDS-PAGE: Coomassie stain of dimeric PS II before (*a*) and after crystallization (*b*) (for details see "Experimental Procedures").

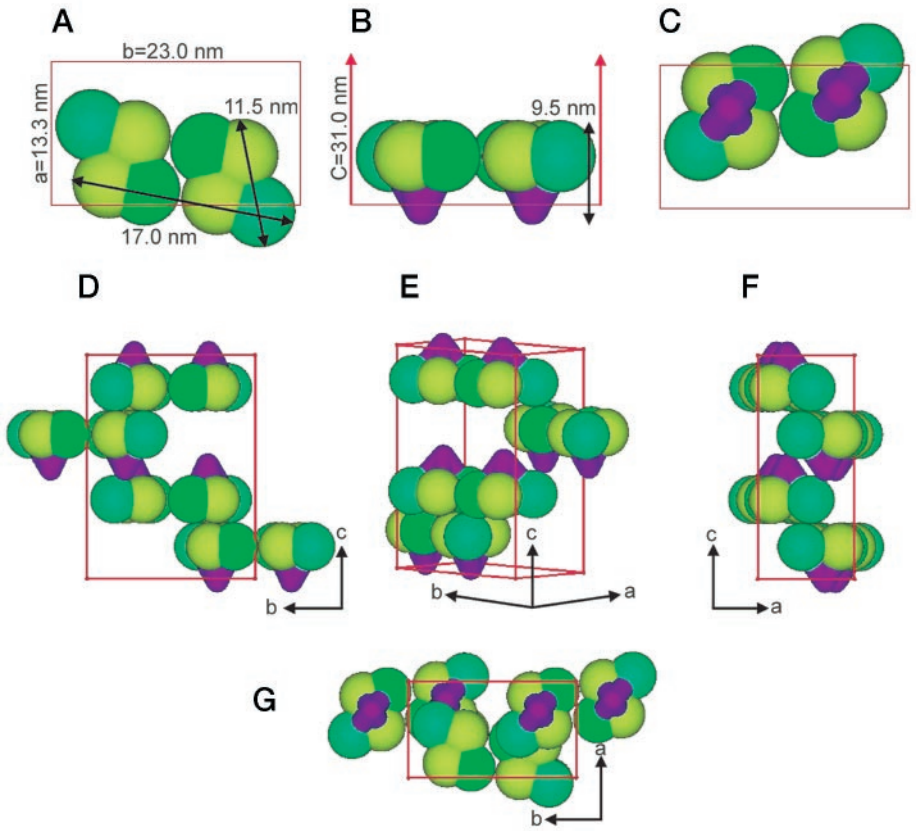

FIG. 7. **Analysis of the cell content and suggested packing in the PS II crystals shown by a simplified sphere model.** The size of the PS II monomer and the dimer formation is chosen to reflect the shape and dimensions of active PS II dimers as seen in electron microscopy images (11, 13). Top view shows a dimeric PS II from the cytoplasma-exposed side (*A*), from the lumen-exposed side (*C*); side view is shown in *B*. The lumen-exposed water-splitting site is shown in *blue*, D1/D2 in *yellow-green*, and CP43/CP47 in *darker green*. *D*, *E*, and *F* display different side views of the suggested packing of four PS II dimers per unit cell; *G* shows a top view of the unit cell.

(41); however, this preparation still contains many contaminating proteins making it unsuitable for structural studies.

Our water-splitting complex also shows a remarkable thermo- and long-term stability, to which both its thermophilic origin and the dimeric structure of PS II preserved by our preparation procedure may contribute. The isolated dimeric complex shows up to 100% higher oxygen rates than monomeric PS II of the same organism (11, 32, 39, 40); comparisons of monomeric and dimeric PS II complexes of mesophiles confirm these observations (9), although at a much lower activity level and considerably less stability.

In addition to the intactness of the electron donor side of our PS II preparation, the acceptor side appears also to be intact and the quinone binding site for Q_B is almost fully occupied. The present work represents, to our knowledge, the first crystallization of PS II with an occupied Q_B site. This is of particular interest, because the Q_B site is the site where herbicides like DCMU and atrazine bind.

PS II from *S. elongatus* is also unique in that it maintains its water-splitting activity even after crystallization: All PS II preparations yielding two- or three-dimensional crystals from other organisms have not been reported to show any oxygen evolution after resolubilization of the crystals. Oxygen-evolving activity was, however, reported of resolubilized three-dimensional crystals that were originally grown from a monomeric PS II preparation of *S. elongatus* and diffracted up to 5 Å (42). Although the activity of this preparation was measured as averaged O_2 flash yield, which is difficult to compare with the oxygen rates measured in our assay, the antenna size of about 60 Chl per (active) reaction center appears high, especially

because values below 40 had been reported before (32). This might also be due to the lower activity of monomeric *versus* dimeric PS II preparations mentioned above.

Although our space group is identical with the one reported for PS II crystals from the same cyanobacterium (42), although with a different type of preparation, we disagree with their suggestion of the packing of the unit cell with four monomeric PS II complexes. Their suggestion is based on a different type of PS II preparation, which has been reported to yield exclusively PS II monomers and to remain monomeric even after crystallization. In consequence, Zouni *et al.* (42) based their analysis on even smaller dimensions for the PS II complex and estimated the specific volume of the PS II crystals to be around 6.8 $Å^3$/Da. Because the dimensions of both unit cells are nearly equal, we propose that both crystals contain overall eight PS II monomers per unit cell, forming four PS II dimers in our case (as verified by size exclusion chromatography of dissolved crystals), which is different from the 4 PS II monomers per unit cell suggested by Zouni *et al.* (42).

In summary, the PS II preparation from the thermophilic cyanobacterium *S. elongatus* reported in this article shows excellent features concerning purity, homogeneity, activity, stability, and quantity. The homogeneous dimeric PS II complexes yielded three-dimensional crystals that diffract up to 4.3 Å, *i.e.* the highest reported resolution to date. The fact that both oxygen-evolving activity and dimeric organization was preserved after resolubilization of these crystals should promote a further structural analysis of PS II, including the water-splitting process in particular and of intrinsic membrane proteins in general. (Structure resolution by multiple isomorphous replacement using heavy atom compounds is in progress.)

Moreover, this thermostable preparation may also facilitate or open new ways of studying PS II as a model for the development of semiartificial, light-driven water-splitting devices.

Acknowledgments— For their support during test beamtime at the ESRF beamlines Grenoble we thank Anastasis Perrakis (ID13), Ed Mitchell (ID14), and the EMBL.

REFERENCES

1. Hankamer, B., Barber, J., and Boekema, E. J. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 641–671
2. Diner, B. A., and Babcock, G. T. (1996) in *Advances in Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., eds) pp. 213–247, Kluwer Academic Publishers, Dordrecht
3. Barry, B. A., and Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7099–7103
4. Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T., and McIntosh, L. (1988) *Biochemistry* **27,** 9071–9074
5. Metz, J. G., Nixon, P. J., Rögner, M., Brudvig, G. W., and Diner, B. A. (1989) *Biochemistry* **28,** 6960–6969
6. Steward, D. H., and Brudvig, G. W. (1998) *Biochim. Biophys. Acta* **1367,** 63–87
7. Hankamer, B., Morris, E. P., and Barber, J. (1999) *Nature Struct. Biol.* **6,** 560–564
8. Zheleva, D., Sharma, J., Panio, M., Morris, H. R., and Barber, J. (1998) *J. Biol. Chem.* **273,** 16122–16127
9. Hankamer, B., Nield, J., Zheleva, D., Boekema, E. J., Janson, S., and Barber, J. (1997) *Eur. J. Biochem.* **243,** 422–429
10. Seidler, A. (1996) *Biochim. Biophys. Acta* **1277,** 35–60
11. Boekema, E. J., Hankamer, B., Bald, D., Kruip, J., Nield, J., Boonstra, A. F., Barber, J., and Rögner, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 175–179
12. Boekema, E. J., Hankamer, B., Nield, J., and Barber, J. (1998) *Eur. J. Biochem.* **252,** 268–276
13. Kuhl, H., Rögner, M., van Breemen, J. F. L., and Boekema, E. J. (1999) *Eur. J. Biochem.* **266,** 453–459
14. Rhee, K.-H., Morris, E.-P., Zheleva, D., Hankamer, B., Kühlbrandt, W., and Barber, J. (1997) *Nature* **389,** 522–526
15. Rhee, K.-H., Morris, E.-P., Barber, J., and Kühlbrandt, W. (1998) *Nature* **396,** 283–286
16. Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985) *Nature* **318,** 618–624
17. Michel, H., and Deisenhofer, J. (1988) *Biochemistry* **27,** 1–7
18. Krauss, N., Schubert, W.-D., Klukas, O., Fromme, P., Witt, H. T., and Saenger, W. (1996) *Nature Struct. Biol.* **3,** 965–973
19. Nakazato, K., Toyoshima, C., Enami, I., and Inoue, Y. (1996) *J. Mol. Biol.* **257,** 225–232
20. Castenholz, R. W. (1969) *Bacteriol. Rev.* **33,** 476–504
21. Lichtenthaler, H. K. (1987) *Methods Enzymol.* **148,** 350–382
22. Thomas, P. E., Ryan, D., and Levin, W. (1976) *Anal. Biochem.* **75,** 168–176
23. Demeter, S., and Vass, I. (1989) *Biochim. Biophys. Acta* **764,** 24–32
24. McPherson, A. (1990) *Eur. J. Biochem.* **189,** 1–23
25. Chayen, N. E., Stewart, P. D. S., and Blow, D. M. (1992) *J. Crystal Growth* **122,** 176–180
26. Yamaoka, T., Satoh, K., and Katoh, S. (1978) *Plant Cell Physiol.* **19,** 943–954
27. Nishiyama, Y., Hayashi, H., Watanabe, T., and Murata, N. (1994) *Plant Physiol.* **105,** 1313–1319
28. Navarro, J. A., Hervas, M., De la Cerda, B., and De la Rosa, M. A. (1995) *Arch. Biochem. Biophys.* **318,** 46–52
29. Shen, J.-R., Ikeuchi, M., and Inoue, Y. (1992) *FEBS Lett.* **301,** 145–149
30. Rutherford, A. W., Crofts, A. R., and Inoue, Y. (1982) *Biochim. Biophys. Acta* **682,** 97–101
31. Matthews, B. M. (1968) *J. Mol. Biol.* **33,** 491–495
32. Dekker, J. P., Boekema, E. J., Witt, H. T., and Rögner, M. (1988) *Biochim. Biophys. Acta* **936,** 307–318
33. Kruip, J. (1999) in *Protein Liquid Chromatography* (Kastner, M., ed) pp. 785–824, Elsevier Science BV, Amsterdam, Netherlands
34. Kruip, J., Karapetyan, N. V., Terekhova, I. V., and Rögner, M. (1999) *J. Biol. Chem.* **274,** 18181–18188
35. Wenk, S.-O., and Kruip, J. (2000) *J. Chromatogr. B.* **737,** 131–142
36. Wenk, S.-O., Boronowsky, U., Peterman, E. J. G., Jäger, C., Amerongen, H. v., Dekker, J. P., and Rögner, M. (1998) in *Photosynthesis: Mechanisms and Effects* (Garab, G., ed) Vol. III, pp. 1537–1540, Kluwer Academic Publishers, Dordrecht, Netherlands
37. Rögner, M., Boekema, E. J., and Barber, J. (1996) *Trends Biochem. Sci.* **21,** 44–49
38. Boussac, A., Kuhl, H., Un, S., Rögner, M., and Rutherford, A. W. (1998) *Biochemistry* **37,** 8995–9000
39. Rögner, M., Dekker, J. P., Boekema, E. J., and Witt, H. T. (1987) *FEBS Lett.* **219,** 207–211
40. Kuhl, H., Krieger, A., Seidler, A., Boussac, A., Rutherford, A. W., and Rögner, M. (1998) in *Photosynthesis: Mechanisms and Effects* (Garab, G., ed) Vol. II, pp. 1001–1004, Kluwer Academic Publishers, Dordrecht, Netherlands
41. Ichimura, T., Miyairi, S., Satoh, K., and Katoh, S. (1992) *Plant Cell Physiol.* **33,** 299–305
42. Zouni, A., Lüneberg, C., Schubert, W. D., Saenger, W., and Witt, H. T. (1998) in *Photosynthesis: Mechanism and Effects* (Garab, G., ed) Vol. II, pp. 925–928, Kluwer Academic Publishers, Dordrecht, Netherlands