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Published in:
European Journal of Biochemistry

DOI:
10.1111/j.1432-1033.1990.tb15458.x

Publication date:
1990

Eur. J. Biochem. 189, 47 – 53 (1990)

Functional and structural analysis of photosystem II core complexes from spinach with high oxygen evolution capacity

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(Received August 30, 1989) – EJB 89 1063

Oxygen-evolving photosystem II core complexes were prepared from spinach by solubilizing photosystem II membrane fragments with dodecyl-β-D-maltoside. The core complexes consist of the intrinsic 47-kDa, 43-kDa, D1 and D2 polypeptides, the two subunits of cytochrome b_{559} and the extrinsic 33-kDa protein. In the presence of 50 mM $CaCl_2$ they exhibit a high oxygen evolution rate of 1.3 ± 0.2 mmol $O_2 \cdot$ mg chlorophyll$^{-1} \cdot h^{-1}$ with either 2,6-dichloro-benzoquinone or $K_3[Fe(CN)_6]$ as acceptor. Electron micrographs of these complexes reveal an obtuse triangular structure in when viewed from the top, measuring 15.3 nm on one side and 10.6 nm on the other two sides. An average height of 7.3 nm was determined from the side view position. These data are in good agreement with previously reported dimensions for photosystem II core complexes [Irrgang, K.-D., Boekema, E. J., Vater, J. and Renger, G. (1988) *Eur. J. Biochem. 178*, 209 – 217]. In contrast to previous reports the extrinsic 33-kDa subunit could be resolved for the first time. It appears as a small protrusion when the complex is viewed from the side and seems to cover the lumenal side of the core complex appearing as a disk with a thickness of 1.5 – 3.3 nm.

In all oxygen-evolving photosynthetic organisms (cyanobacteria, algae and higher plants) the key steps in water oxidation take place in a thylakoid-membrane-bound complex referred to as photosystem II. The overall reaction sequence of photosystem II is energetically driven by photooxidation of a special chlorophyll *a* (P680), with pheophytin as the primary acceptor, and subsequent electron transfer to a specifically bound plastoquinone molecule (Q_A). The latter step is required for stabilization of the primary charge separation sufficient to permit water cleavage [1]. The holes produced at $P680^+$ lead to oxidation of water to dioxygen via a four-step univalent redox-reaction sequence at a manganese-containing catalytic site; whereas the electrons of Q_A^- reduce mobile plastoquinone to the quinol form via a two-step univalent mechanism at a specific binding site denoted as the Q_B site (for recent reviews see [2 – 4]). Based on different lines of evidence, all functional redox groups of photosystem II are currently assumed to be incorporated into a heterodimer of two polypeptides designated as D1 and D2, each consisting of about 350 amino acid residues. This assignment would imply that water oxidation would also occur within this complex. D1 and D2 exhibit remarkable sequence analogies with the subunits L and M of purple-bacteria reaction centers (for review see [5, 6]), but in contrast to the situation for purple-bacteria reaction centers, the detailed structure of the D1/D2 heterodimer remains unresolved.

One key problem is the failure to develop a suitable procedure which permits the preparation of functionally active D1/D2 complex. All photosystem II reaction center complexes isolated so far contain, in addition to D1/D2, the two subunits of cytochrome b_{559} and probably at least one further subunit of small relative molecular mass [7 – 10]. Such preparations lack Q_A and the manganese cluster at the catalytic site of water oxidation. On the other hand, photosystem II core complexes with high oxygen evolution capacity have been isolated but they are markedly bigger, containing at least seven polypeptides and a significant number (50 – 100) of antenna pigments [11 – 14]. These circumstances highly complicate the structural analysis of photosystem II.

In this study we report on the isolation of photosystem II core complexes from higher plants which exhibit high rates of oxygen evolution. They contain at least seven polypeptides and are characterized by molecular masses of 250 – 300 kDa. The structure of these complexes is analyzed by electron microscopy.

MATERIALS AND METHODS

Preparation of oxygen-evolving photosystem II core complexes

Oxygen-evolving photosystem II core complexes were extracted from photosystem II membrane fragments, isolated according to the method of Berthold et al. [15], with slight modifications as described by Völker et al. [16]. Extraction of the oxygen-evolving core complexes was achieved by a two-step mild solubilization with the detergent dodecyl-β-D-maltoside. The whole preparation procedure was carried out in the dark and on ice. First, photosystem II membrane fragments were incubated for 1 h with a detergent/chlorophyll ratio of 10:1 and 350 mM NaCl in 10 mM $CaCl_2$, 0.3 M sucrose, 30 mM Mes/NaOH, pH 6.5 (buffer A) with moderate stirring. The chlorophyll concentration during this first step was 1.5 mg/ml. Second, the suspension was diluted 1:2 with buffer A, to decrease the chlorophyll concentration to 0.5 mg/ml, then incubated for another hour. During this second incubation period, the solubilized photosystem II membrane fragments were centrifuged for 15 min at $42500 \times g$ to remove unsolubilized material. The supernatant was loaded onto a

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Abbreviations. Q, plastoquinone; CP29, 29-kDa protein.

sucrose density gradient of 20% – 40% sucrose in 0.025% dodecyl-β-D-maltoside, 10 mM $CaCl_2$, 30 mM Mes/NaOH, pH 6.5 (buffer B) which was then centrifuged in a Beckman VTi 50 rotor at 210000 × *g* for 10 h at 4°C.

Electrophoretic and chromatographic analysis

The polypeptide composition was determined by discontinuous urea/SDS/PAGE (6% stacking gel, 14% resolving gel) with 5 M urea and 0.1% SDS [17, 18]. Applied samples were previously incubated for 1 h at room temperature in 1% SDS, 5% 2-mercaptoethanol and 62.5 mM Tris/HCl pH 6.8. The gels were both Coomassie blue and silver stained [19].

Gel filtration was carried out in buffer B on a Superose 6 FPLC column (Pharmacia/LKB) with a flow rate of 0.5 ml/min. Prior to the experiments the column was calibrated with standard proteins from a calibration kit (Pharmacia/LKB). The protein standards used were thyroglobulin (330-kDa subunit), ferritin (440-kDa and 220-kDa subunits), aldolase (158 kDa) and chymotrypsinogen (25 kDa).

Spectroscopic analysis

Absorption spectra were recorded with a Beckmann double-beam spectrophotometer (Model 35) at room temperature. The content of cytochrome b_{559}/reaction center was calculated from the reduced-minus-oxidized difference spectra. Samples with a chlorophyll concentration of 0.12 mM were oxidized in the presence of 1 mM $K_3[Fe(CN)_6]$ and then converted into the reduced form by incubation with 10 mM dithionite for 30 min. The difference spectra were recorded from 540 – 590 nm on a Shimadzu double-beam spectrophotometer (model UV 3000).

Assay of oxygen evolution

Oxygen evolution was measured with a Clark-type electrode at 20°C. The water-cooled cuvette contained a suspension of photosystem II core complexes (5 μM chlorophyll), 50 mM $CaCl_2$, 10 mM NaCl and 20 mM Mes/NaOH pH 6.5 with 1 mM $K_3[Fe(CN)_6]$ or 2,6-dichlorobenzoquinone as electron acceptors. Light was provided by an Osram Xenophot HLX 64653 lamp (24 V, 250 W). When indicated, different salt concentrations or other pH values were used.

Electron microscopy

A fresh preparation of photosystem II core complexes was diluted to a chlorophyll concentration of about 5 μg/ml with buffer C (10 mM Mes/NaOH pH 6.5) containing 0.015 – 0.03% dodecyl-β-D-maltoside. Specimens were prepared by the droplet method, using uranyl acetate as a negative stain. Electron microscopy was carried out on a Philips EM 400T electron microscope equipped with a low-dose unit at a principal magnification of 60000. Micrographs were digitized with a Joyce Loebl Scandig densitometer. The scanning step used was 50 μm, corresponding to a pixel (image element) size of 0.83 nm on the specimen level. Image analysis was performed within the framework of the IMAGIC software system on a Micro-VAX computer (Digital Equipment Corporation) [20].

RESULTS AND DISCUSSION

After centrifugation two distinct and well-separated green bands appeared in the density gradient. They were both analyzed for protein and chlorophyll content and composition (Fig. 1). Up to 80% chlorophyll was retained by the upper band consisting of light-harvesting complex II with a chlorophyll *a*/chlorophyll *b* ratio of 1.3 and a protein/chlorophyll ratio of 2.5. The remaining chlorophyll was collected with the lower green band which contained purified photosystem II core complexes. In contrast to the light-harvesting complex II fraction, the core complex fraction was enriched both in chlorophyll *a* and in protein. The chlorophyll *a*/chlorophyll *b*

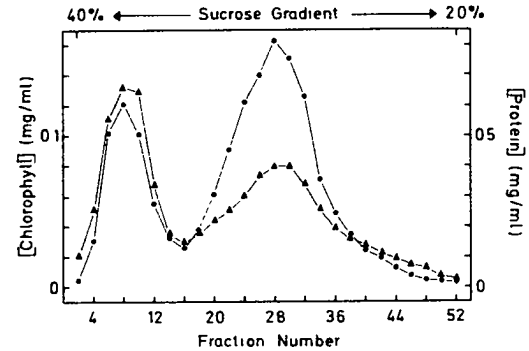

Fig. 1. *Chlorophyll (●) and protein (▲) profiles of the fractionated sucrose density gradient*

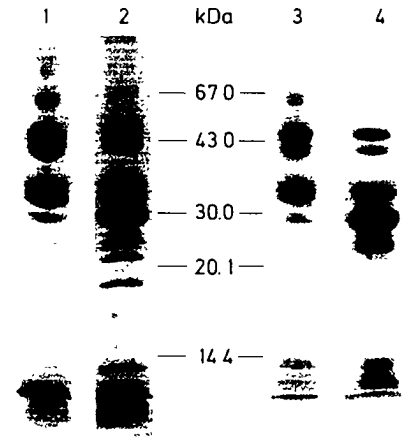

Fig. 2. *Urea/SDS/PAGE of photosystem II core complexes (lanes 1 and 3) and photosystem II membrane fragments (lanes 2 and 4).* Lanes 1 and 2 were Coomassie blue stained (50 μg protein applied); lanes 3 and 4 were silver stained (30 μg protein applied)

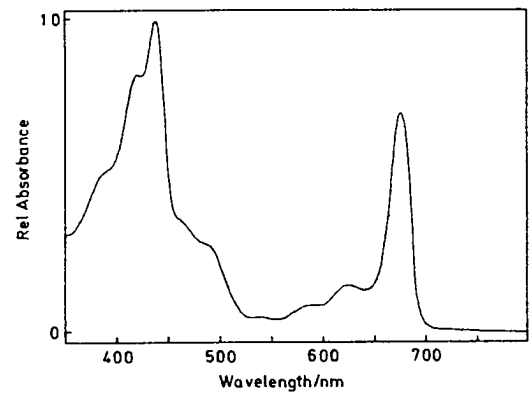

Fig. 3. *Room-temperature absorption spectrum of photosystem II core complexes*

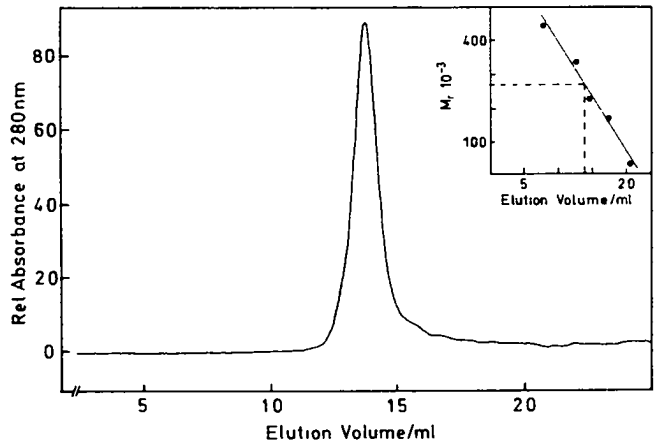

Fig. 4. *Elution profile of the FPLC gel filtration of photosystem II core complexes (200 μg protein applied).* The calibration diagram and the estimation of the relative molecular masses of the photosystem II core complexes are shown in the insert

ratio increased to six, the protein/chlorophyll ratio was 5–6. Electrophoretic analysis of the purified photosystem II core complex revealed polypeptides with molecular masses (kDa) of 47, 43, 34, 33, 31, 9 and 4, and traces of a 29-kDa protein (CP 29) (Fig. 2). However, most CP29 remained in the light-harvesting complex II fraction. Further, it should be emphasized that none of the core proteins appeared in the light-harvesting complex fraction and practically no light-harvesting complex II proteins were found in the core fraction. There may perhaps be a slight heterogeneity of photosystem II core populations concerning the presence of CP29. As CP29 is a chlorophyll *a/b* binding protein this could also explain a small remaining part of chlorophyll *b* observed in the absorption spectrum and indicated by a shoulder in the region of 460–480 nm (Fig. 3). On the other hand, FPLC gel-filtration experiments did not reflect the possibility of two different photosystem II core populations. The elution profile showed one single peak. Based on the calibration diagram the total particle size was estimated to be around 250–300 kDa (Fig. 4). If there is heterogeneity, the difference in molecular mass between the two populations of photosystem II core complexes is either not big enough to result in two distinct elution components or the population containing CP29 is too small to be detected.

The reduced-minus-oxidized difference spectra of the core complexes exhibited a pronounced band peaking at 559 nm which is assigned to cytochrome b_{559} (data not shown). Using a molecular extinction coefficient of 15000 M^{-1} cm^{-1} [21] quantitative calculations yielded 40–50 mol chlorophyll/mol cytochrome b_{559}. Based on comparative measurements of the average oxygen yield/flash leading to a ratio of 90 ± 10 chlorophyll molecules/reaction center, a stoichiometry of two cytochromes b_{559} molecules/reaction center is obtained. This corresponds well to the various data reported previously [22–24] and is in agreement with an assumed dimeric form of cytochrome b_{559}. However, there is still a controversy concerning the number and arrangement of subunits of cytochrome b_{559}/reaction center [25].

To check the activity of the photosystem II core preparations, various measurements of the oxygen evolution rate were performed. First, experimental conditions for maximum activity were tested. The pH dependence exhibited a broad maximum peaking around pH 6.5 which was practically invariant with the use of different exogenous electron acceptors (data not shown).

Various electron acceptors were analyzed for their efficiency. In general, the highest rates of oxygen evolution (maximum value, 1700 μmol O_2 · mg chlorophyll^{-1} · h^{-1}; average value 1300 ± 200 μmol O_2 · mg chlorophyll^{-1} · h^{-1}) were measured either with $K_3[Fe(CN)_6]$ or 2,6-dichlorobenzoquinone under saturating light excitation and in the presence of 50 mM $CaCl_2$. It should be mentioned that in some preparations the highest rates were obtained with $K_3[Fe(CN)_6]$, in others with 2,6-dichlorobenzoquinone. Phenyl-*p*-benzoquinone also supported a high electron-transport activity, whereas 2,6-dichloroindophenol was found to be a rather poor acceptor giving a maximum rate of only 5–20% of that measured with $K_3[Fe(CN)_6]$ (data not shown). A comparable poor efficiency of 2,6-dichloroindophenol was recently observed for photosystem II complexes isolated from a chlorophyll-*b*-deficient rice mutant by octyl-β-D-glucoside solubilization [26]. However, in contrast to our results $K_3[Fe(CN)_6]$ also acted as a rather poor acceptor for these photosystem II complexes. This difference probably reflects a different extent of modification in the environment of the Q_B site due to detergent treatment. This part of the photosystem II complex is obviously the most susceptible region during the solubilization procedure as previously shown by herbicide-binding studies of photosystem II membrane fragments [27]. However, it should be emphasized that we observed a remarkable 3-(3,4-dichlorophenyl)-1,1-dimethylurea sensitivity indicating that the Q_B site is not severely damaged in our photosystem II core preparation. In the presence of 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea the oxygen evolution rate was largely diminished (70% inhibition).

A strongly stimulating effect on the oxygen evolution rate was exerted by $CaCl_2$ which could not be substituted by $MgCl_2$. This finding corresponds to results reported previously [23]. It was assumed to be caused by $CaCl_2$-induced protection against photoinhibition. However, several lines of evidence indicate that this is not the only explanation. The extent of $CaCl_2$ stimulation depended strongly on the electron acceptor used. In particular, the measurements with $K_3[Fe(CN)_6]$ were influenced by $CaCl_2$ (Fig. 5). The rate of oxygen evolution obtained without $CaCl_2$ was only one-third of that measured in the presence of 50 mM $CaCl_2$. An effect of the same order was not observed if the measurements were performed with 2,6-dichlorobenzoquinone as acceptor. However, there seems to be a superimposition of at least two different phenomena. At very low $CaCl_2$ concentrations (less

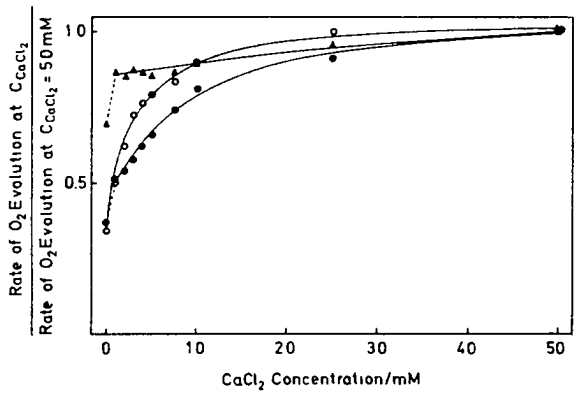

Fig. 5. *Normalized oxygen evolution rate as a function of $CaCl_2$ concentration in the presence of 2,6-dichlorobenzoquinone (▲) and $K_3[Fe(CN)_6]$ (●).* Oxygen evolution rates of photosystem II core complexes isolated in the absence of $CaCl_2$ were measured with $K_3[Fe(CN)_6]$ as acceptor and are symbolized by (○). The curves for measurements with $K_3[Fe(CN)_6]$ were calculated and revealed a K_m value of 8.9 mM for photosystem II core complexes prepared in the presence of $CaCl_2$ and a K_m value of 2.9 mM for photosystem II core complexes prepared in the absence of $CaCl_2$

than 1 mM) a stimulation could be observed regardless of the electron acceptor used. This is indicated by the dotted lines in Fig. 5. For higher $CaCl_2$ concentrations only the oxygen evolution rates measured in the presence of $K_3[Fe(CN)_6]$ revealed a significant stimulation which could be described by a typical hyperbolic saturation curve. The results suggest that conformational changes on the acceptor side may be involved at higher $CaCl_2$ concentrations.

For further investigations of possible Ca^{2+} effects, some photosystem II core complexes were isolated without addition of $CaCl_2$ to the buffers used during the preparation. For these specially prepared core complexes, an even stronger stimulating effect of $CaCl_2$ could be observed in the presence of $K_3[Fe(CN)_6]$. The K_m value was one-third of the K_m value for normally prepared core complexes (Fig. 5). However, the absolute rate of oxygen evolution of core complexes prepared in the absence of $CaCl_2$ was less than 50% of the rate of normally prepared core complexes for each $CaCl_2$ concentration in the assay medium. This indicates that isolation in the absence of Ca^{2+} leads to a functional blockage of a fraction of photosystem II core complexes which cannot be reactivated by the addition of $CaCl_2$ to the assay medium. On the other hand, photosystem II core complexes prepared and measured in the complete absence of $CaCl_2$ still revealed a small but noticeable oxygen evolution rate (250 μmol O_2 · mg chlorophyll^{-1} · h^{-1}) which suggests that part of the Ca^{2+} required for water oxidation is very tightly bound and is not released during the preparation procedure. The idea of tightly bound Ca^{2+} is in line with recent findings of Shen et al. [28].

Moreover, the $CaCl_2$-dependent stimulation of the oxygen evolution rate could not be assigned a protective role against photoinhibition. The oxygen evolution rate decreased to 80% during a second illumination period (2 × 10 s) regardless of the actual $CaCl_2$ concentration (data not shown). No protective effect at higher $CaCl_2$ concentrations could be found.

The above results show that the photosystem II core complexes are well-characterized as regards their biochemical and functional properties. They exhibit a very high oxygen evolution capacity and should provide a suitable material for structural investigations.

The structure of the oxygen-evolving photosystem II core complexes was analyzed by electron microscopy. Two different projections were mainly observed: a top and a side view (Fig. 6). Frequently core particles diluted in buffer plus dodecyl-β-D-maltoside revealed an aggregation of two complexes, mostly with their smallest dimension (side view) as the repeating unit (Fig. 6). The aggregation is probably due to the lower detergent and salt concentrations during specimen preparation, since the gel filtration exhibited a homogeneous population of monomeric particles (see Fig. 4).

The top view projection was an obtuse triangle with a length of 15.6 ± 1.0 nm (n = 200) and a width of 10.6 ± 1.0 nm (n = 54). The value for the length was derived from particles in both top and side views. In fact, particles in the side view appeared to be more extended, by about 0.3 nm, perhaps due to a slightly different arrangement of the detergent molecules. It is interesting to note that similarly shaped projections (15.1 × 10.7 nm) have previously been found for spinach photosystem II core complex preparations that lack any oxygen evolution activity and are completely depleted of the extrinsic subunits [22]. This findings leads to the conclusion that photosystem II core complexes exhibit almost identical top view projections regardless of the oxygen evolution activity and presence of the extrinsic 33-kDa subunit. However, it has to be mentioned that the triangular projection, with dimensions of 13 nm × 13 nm previously reported for complexes without the extrinsic 33-kDa subunit, was scarcely observed in oxygen-evolving core complexes.

Depending on the dodecyl-β-D-maltoside concentration during specimen preparation the particles were more or less aggregated. As a striking phenomenon in both cases, i.e. in single or aggregated forms, particles with a small protrusion could be seen occasionally (Fig. 7). This feature of structurally resolved protrusions has never been observed in photosystem II core complexes lacking oxygen evolution and the extrinsic 33-kDa polypeptide. It therefore appears very likely that the protrusions are due to the presence of the extrinsic 33-kDa subunit.

The thickness of the particles could be determined from the side view. To calculate the overall thickness, particles in the single form and without a visible separate protrusion were selected and the stain-excluding regions were measured. From 51 photosystem II complexes, an overall thickness of 7.3 ± 0.5 nm was obtained. This value is slightly larger than that determined in our previous work on spinach core complexes deprived of oxygen evolution capacity [22]. It is interesting to note that these particles exhibited markedly different aggregation features. They formed long strings. The thickness of these photosystem II core complexes was measured as a center-to-center distance of 6.8 nm. The presence of the extrinsic 33-kDa subunit in the oxygen-evolving photosystem II core complexes obviously prevents the string formation.

The dimensions of the protrusion which is ascribed to the extrinsic 33-kDa polypeptide could be better recognized in the aggregated dimeric form. They were determined to be 3.3 ± 0.3 nm and 7.2 ± 1.4 nm (n = 30). Some dimeric aggregates which had the protrusion localized at about the same position could therefore be computer-averaged by bringing them into equivalent position by alignment procedures. As can be seen in Fig. 8, the protrusion makes particles of 5.8 nm thickness about 3.3 nm larger. Since the height of the protrusion is quite small and close to what can be seen by direct visualization in the electron microscope, it is likely that these

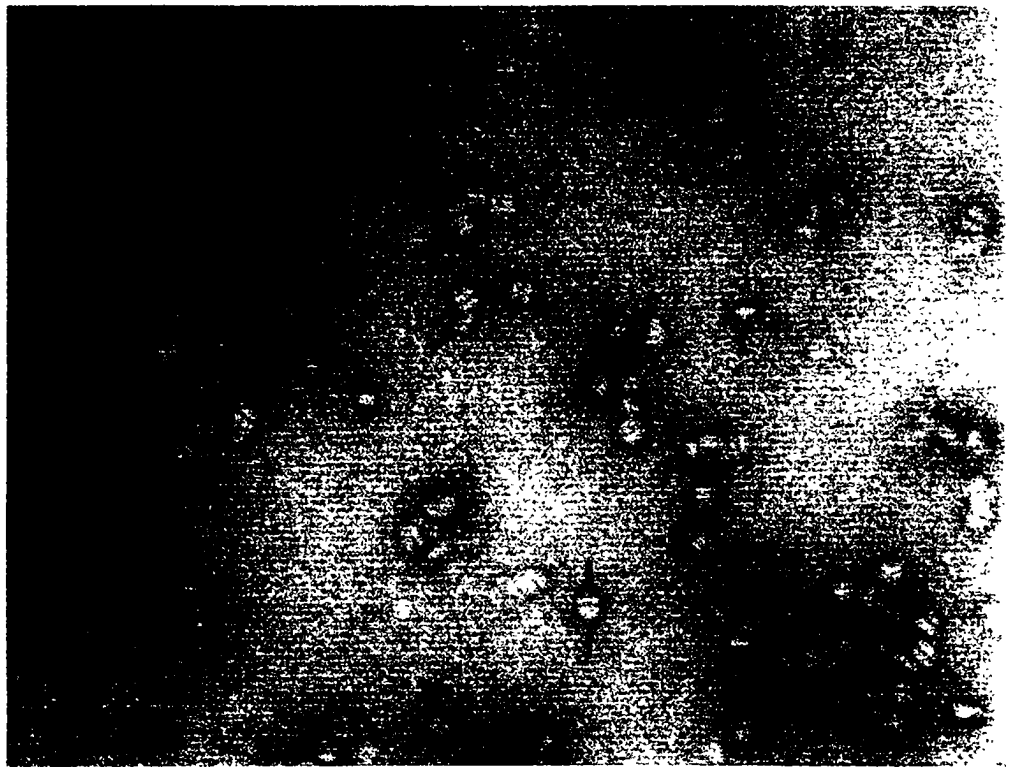

Fig. 6. *Electron micrograph of dodecyl-β-D-maltoside-solubilized photosystem II core complexes from spinach.* Thick arrows indicate top view projections; thin arrows indicate side view projections with visible protrusion

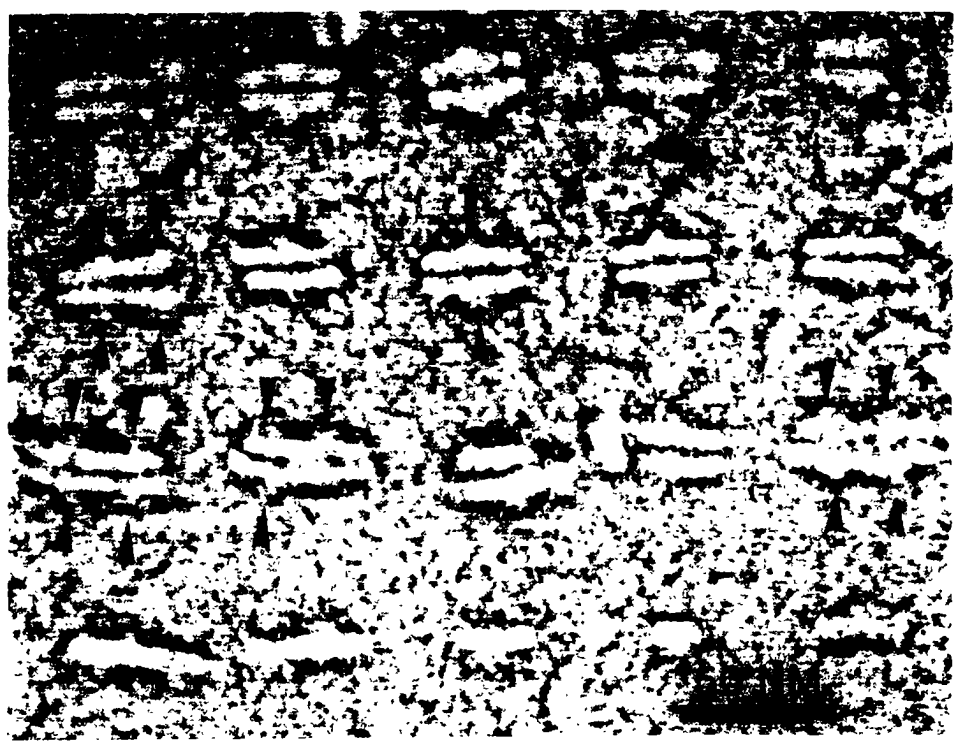

Fig. 7. *Selected parts of digitalized electron micrographs showing aggregated photosystem II core complexes in side-view position revealing the 33-kDa subunit as a small protrusion (arrows).* The complexes are artificially aggregated face to face with the stroma-exposed sides

dimensions are slightly exaggerated, while smaller looking protrusions are not always recognized.

Based on the results of the present study it can be concluded that the photosystem II core complex of spinach has the shape of an obtuse triangle with dimensions of 15.3–10.6 nm in the top view and an overall height of 7.3 nm. The overall (average) height of the particle is, however, not the same as the maximum height since part of the oxygen-evolving

Fig. 8. *Averaged image of 14 aligned photosystem II core complex dimers.* Some of these aggregates are shown in Fig. 7 (first four in the upper row)

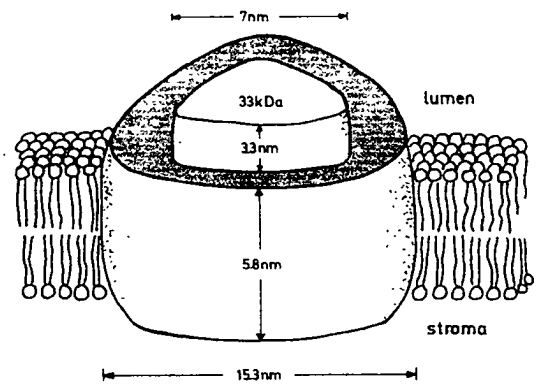

Fig. 9. *Model of an oxygen-evolving photosystem-II core complex from spinach.* The membrane-integral part of the core complex has an obtuse triangular shape and is covered by the 33-kDa subunit at the lumenal side (for details see Results and Discussion). The dimensions of the protrusion are from complexes where it could be clearly recognized; therefore the size may be slightly overestimated

photosystem II core complexes is covered by a disk-like protrusion thought to be the extrinsic 33-kDa subunit. Taking into account a measured thickness of 5.8 nm without protrusion (Fig. 8) and an overall (average) height of 7.3 nm (with a protrusion that is not always visible), it can be deduced that the actual height of the protrusion must be somewhere between 1.5 nm and 3.3 nm. The results are presented in the form of a model in Fig. 9. The basic idea of this model (localization of the extrinsic proteins at the lumenal side) corresponds well to the previously published work of Seibert et al. [29].

The presented data are in accordance with other work. Monomeric photosystem II particles from the cyanobacterium *Synechococcus* sp. have dimensions of 15.4×10.8 nm measured for the top view in dodecyl-β-D-maltoside and a height of 7 nm [30]. On the other hand, photosystem II core complexes with high oxygen evolution activity from cyanobacteria and spinach differ slightly but significantly in the shape of their top view projections. Photosystem II particles of the cyanobacterium are elongated and oval, whereas spinach photosystem II particles are more obtuse triangular complexes. The triangular shape was already observed for spinach photosystem II core complexes completely deprived of any oxygen evolution capacity and the extrinsic regulatory polypeptides. It was also found to be independent of the detergent used during the preparation procedure. In a comparative study, photosystem II core complexes from spinach which were solubilized either with dodecyl-β-D-maltoside or octylglucoside revealed a triangular shape [22]. Therefore, the present results support the idea that the different top view shape of photosystem II in cyanobacteria and higher plants relates to the highly different structural organization of the antenna systems as explicitly outlined in our previous study [22]. Furthermore, not only the shape but also the organization form of photosystem II may differ for cyanobacteria and higher plants. A dimeric form of photosystem II is suggested for cyanobacteria [30, 31]. In contrast, dimers of photosystem II core complexes from spinach attached at their membrane-exposed sides were scarcely seen on electron micrographs. The majority of observed dimers were artificially aggregated face to face with their stroma sides. There is obviously a tendency for detergent-induced face-to-face aggregation during the preparation. This leads to string formation if all extrinsic subunits are missing and to dimer formation in the presence of the 33-kDa extrinsic subunit. There is not sufficient evidence for a dimeric organisation form *in vivo* for photosystem II of higher plants.

The authors would like to thank M. Müller for skilful technical assistance. Financial support by European Rescue *Program-Sondervermögen* (ERP 2603), *Deutsche Forschungsgemeinschaft* (sfb 312) and *Fonds der Chemischen Industrie* is gratefully acknowledged.

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