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Structural determination of the photosystem II core complex from spinach

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A photosystem II core complex was purified with high yield from spinach by solubilization with β-dodecylmaltoside. The complex consisted of polypeptides with molecular mass 47, 43, 34, 31, 9 and 4 kDa and some minor components, as detected by silver-staining of polyacrylamide gels. There was no indication for the chlorophyll-a/b-binding, light-harvesting complex polypeptides. The core complex revealed electron-transfer activity (1,5-diphenylcarbazide → 2,6-dichloroindophenol) of about 30 μmol reduced 2,6-dichloroindophenol/mg chlorophyll/h. The structural integrity was analyzed by electron microscopy. The detergent-solubilized protein complex has the shape of a triangular disk with a maximum diameter of 13 nm and a maximum height of 6.8 nm. The shape of this core complex differs considerably from that of cyanobacterial photosystem II membrane fragments, which are elongated particles. The structural differences between both the complexes of higher plants and cyanobacteria are discussed with special emphasis on their association with the antenna apparatus in the photosynthetic membranes.

Light-induced photosynthetic water oxidation and plastoquinone reduction take place in photosystem II (PS II) of higher plants, algae and cyanobacteria [1, 2]. The overall reaction is energetically driven by photooxidation of (a) special chlorophyll (Chl) molecule(s) (symbolized by P680) and concomitant electron transfer to a plastoquinone called Q_a via pheophytin a as intermediate. The functional groups P680, pheophytin a and Q_a are anisotropically bound to transmembrane polypeptides referred to as PS II reaction center complex. The nature of its apoprotein was a matter of debate during the last half decade and some aspects of this problem are still not satisfactorily resolved. Low-temperature fluorescence emission studies led Nakatani et al. to conclude that the so-called CP47 protein is the apoprotein of the reaction center and a 40-kDa chlorophyll, a binding polypeptide (symbolized in the literature as CP43), acts as a core antenna [3]. On the other hand, results on the functional and structural organization of purple bacteria reaction centers revealed remarkable sequence similarities between the L and M subunits and two intrinsic membrane proteins of the PS II [4–12], named D1 and D2 (D because of the diffuse nature of the bands which the proteins form on polyacrylamide gels [13]). The crystallization of the reaction center of *Rhodopseudomonas viridis* [14] and the subsequent crystallographic analysis resulted in the detailed structure of the polypeptide subunits and the arrangement of the prosthetic groups at atomic resolution [15, 16].

This information on a bacterial reaction center, in combination with the DNA sequence data for the genes of the D1 and D2 proteins supported the view of the homology between the reaction centers of *Rhodobacter* and PS II of higher plants. Taking into account functional similarities [17], Michel and

Deisenhofer [12] and Trebst et al. [18, 19] proposed that D1 and D2 are the PS II reaction center binding proteins, rather than CP47. In this model D1 corresponds to the L subunit and D2 to the M subunit. The recent identification of Chl-a binding polypeptides in the 30-kDa range [20, 21] and the isolation of a D1-D2-cytochrome b_{559} complex [22, 23], which exhibits characteristics typical for formation and decay of a primary radical pair (P680$^+$ pheophytine$^-$ [24–26]) gave strong experimental support for this proposal. Unfortunately a stabilization of the primary charge separation could not be achieved, because this preparation lacks Q_a, in contrast to the isolated LM complex of purple bacteria [27]. A functionally complete PS II reaction center has previously only been obtained in so-called PS II core complexes containing CP47 in addition to D1, D2 and cytochrome b_{559} [28–30].

Structural informations on the PS II core as well as the reaction center complex are scarce at the moment. Until recently, integral membrane proteins of chloroplast thylakoids had been merely studied by low-resolution freeze fracture and freeze-etching electron microscopy. A more detailed picture of the three-dimensional structure of thylakoid membrane complexes is only available for the light-harvesting complex (LHC) II, which has been obtained in crystalline sheets (see [31] for a review and [32]). Lately, Rögner et al. determined the size and shape of the O_2-evolving complex from *Synechococcus* sp. [33]. In this report we have investigated the biochemical and biophysical properties of the PS II core complex from spinach and analyzed its structural integrity by electron microscopy.

MATERIALS AND METHODS

Isolation of PS II

PS II membrane fragments were isolated from spinach chloroplasts according to Berthold et al. [34] with modifications as indicated in [35] and addition of protein inhibitors as described (*vide infra*).

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Abbreviations. Chl, chlorophyll; LHC, light-harvesting complex; P-680, reaction center chlorophyll a molecule of photosystem II; Q_a and Q_b, primary and secondary quinone acceptors; RC, reaction center; PS II, photosystem II.

Preparation of PS II intrinsic membrane proteins

Purified PS II membrane fragments were depleted of extrinsic proteins by a high-salt washing procedure (for details see Fig. 1) in buffer A (40 mM Mes/NaOH pH 6.5, 0.3 M sucrose, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine) at $0-4°C$. The last pellet obtained after washing the membrane fragments with 2 M NaCl contained no detectable extrinsic polypeptide on silver-stained gels.

Isolation of the PS II core complex

The core complex has been isolated by a modified method according to Westhoff et al. [36]. The intrinsic membrane proteins were resuspended in buffer B (10 mM Mes/NaOH pH 6.5, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine) and β-dodecylmaltoside was added to a detergent/Chl ratio of 10:1. Solubilization was carried out for 1 h at 4°C in the dark. Non-solubilized protein was eliminated by a low-speed centrifugation in a Beckmann minifuge for 10 min (12000 rpm), 4°C) in the dark. The supernatant was loaded onto a linear sucrose gradient of $10-30\%$ (mass/vol.) in buffer B in the presence of 0.05% (mass/vol.) β-dodecylmaltoside $(0.3-0.5$-ml aliquots of $0.03-0.5$ mg Chl/tube). Centrifugation was carried for 20 h (35000 rpm, $2-4°C$) in a Beckmann SW 41 rotor. The tubes were pierced and fractions of approximately 0.5 ml collected. The gradients were analyzed for protein and Chl distributions [37, 38]. Sucrose content was determined refractometrically.

Electrophoretical analysis

Aliquots of gradient fractions were mixed with a proportional volume of the sample buffer as in [20] in order to analyze the polypeptide composition on SDS/urea/polyacrylamide gels (6% stacking, 14% resolving gel). Both gels contained 0.1% (mass/vol.) SDS and 5 M urea. Silver staining was performed according to Oakley et al. [39].

Immunoblotting

Immunologically reactive D1 and D2 polypeptides were identified using the blot procedure of Towbin et al. [40] in combination with the staining technique developed by Bode et al. [41]. Anti-D2 was a generous gift of Prof. Trebst (Bochum) and anti-D1 was raised in a rabbit using electrophoretically purified antigen.

Spectroscopic measurements

Absorption spectra were recorded at room temperature using a Perkin Elmer double-beam spectrophotometer (model 556) equipped with a baseline corrector (Hitachi Ltd). Cytochrome b_{559} was determined as described by Hodges et al. [42] from the dithionite-ferricyanide difference spectra.

Fluorescence emission spectra at 77 K have been measured with a commercial fluorimeter SLM 4800S (SLM Instr. Urbana, USA). The samples were frozen in liquid nitrogen and dark-adapted for 5 min before excitation with blue light of $\lambda = 435$ nm (half-width 16 nm). Emissions (half-width 4 nm) were scanned between $600-750$ nm. Second-derivative spectra were calculated using a home-made computer program.

Electron transfer measurements

As a measure of the PS II core complex electron-transfer activity the photoreduction of 2,6-dichloroindophenol with 1,5-diphenylcarbazide as a donor was spectrophotometrically monitored at 590 nm (Eppendorf photometer). Samples were illuminated in a water-cooled cuvette by light from a 500-W xenon lamp (Osram) passing a red filter (Schott RG 610/2). The reaction mixture for the dichloroindophenol reduction contained 5 μM Chl in buffer B with 40 μM dichloroindophenol and 500 μM 1,5-diphenylcarbazide.

Electron microscopy

Immediately before use purified PS II core complexes were dialyzed (dialysis cut-off, 2 kDa; Sigma) against buffer B containing $0.015-0.03\%$ (mass/vol.) β-dodecylmaltoside and 20 mM NaCl and finally concentrated either by ultrafiltration on PM 10 membranes with an Amicon 8MC ultrafiltration system or by vacuum centrifugation (Savant) in the dark.

Specimens for electron microscopy were prepared by the droplet method, using uranyl acetate as a negative stain. Electron microscopy was carried out on Philips EM 300 and EM 400T electron microscopes at $50-70 \times 10^3$-fold magnification. Micrographs were digitized with a Datacopy Model 610F electronic digitizing camera [43]. The scanning step used was 32 μm, corresponding to a pixel (image element) size of 0.47 nm on the specimen level. Image analysis was performed within the framework of the IMAGIC software system [44] on a MicroVAX computer (Digital Equipment Corporation).

All chemicals used were of the highest purity available. β-Dodecylmaltoside was from Boehringer (Mannheim); Triton X-100, Tween 20, benzamidine and phenylmethylsulfonyl fluoride were from Sigma; and electrophoresis chemicals were from LKB. Nitrocellulose membranes (0.45 μm) were from Schleicher and Schüll, fast red and naphtol AS X phosphate from Sigma. Alkaline phosphatase conjugated to goat anti-(rabbit IgG) antibody was purchased from Medac (Hamburg).

RESULTS AND DISCUSSION

Biochemical characterization of the core complex

PS II intrinsic membrane proteins, isolated as demonstrated in the flow diagram of Fig. 1, were solubilized with β-dodecylmaltoside and separated by detergent-containing sucrose density gradients. PS II core fractions, depleted of LHC polypeptides, were pooled and analyzed for protein composition and Chl content (see Fig. 2). Analysis by SDS/urea/polyacrylamide gel electrophoresis, in combination with a highly sensitive silver stain technique, showed that the isolated core complex consisted of polypeptides of 47, 43, 34, 31, 9 and 4 kDa and some minor components in negligible concentrations. It should be mentioned that we did not observe any of these latter components on Coomassie-R-250-stained gels (Fig. 3A and B). Using both techniques there was no indication for any polypeptide of the mobile LHC II, which otherwise migrated in the region of $26-29$ kDa in our gel system [20] (see Fig. 3A and B). Although the whole preparation was carried out in the presence of two protease inhibitors (see Materials and Methods) we cannot entirely exclude degradation products of higher-molecular-mass components. The presence and integrity of the two reaction center (RC) polypeptides D1 and D2 have, therefore, also been checked by
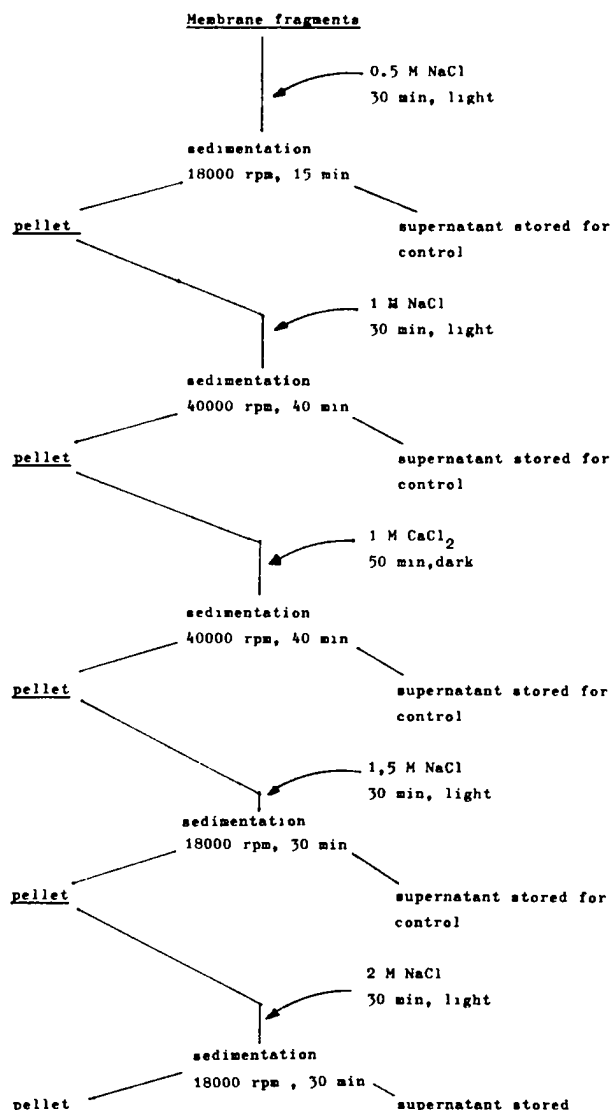
Fig. 1. *Flow diagram for the preparation of the PS II intrinsic membrane protein complexes*

Fig. 2. *β-Dodecylmaltoside sucrose density centrifugation of PS II intrinsic membrane protein complexes.* (----) Refractometrical determination of sucrose; (———) chlorophyll distribution

immunoblotting. Both proteins were clearly detected and no proteolytic cleavage products found (Fig. 3). The D1 and D2 proteins were identified as the bands migrating at 34 kDa and approximately 31 kDa. Both proteins tended to form dimers (Fig. 4), which is in agreement with a recent immunological analysis of the PS II reaction center [45].

A quantitative calculation of the Chl content of the PS II core complex revealed about 40 Chl a molecules/complex. The presence of cytochrome b_{559} was proven by dithionite − ferricyanide difference spectra. We determined approximately 2 mol cytochrome $b_{559}/40$ mol Chl. The rather low Chl content is presumably due to some loss of the 47-kDa and 43-kDa proteins, which were also detected in the LHC fractions (Fig. 3). In contrast to this, the D1 and D2 proteins could only be identified in the core complex. The yield of the core complex was 300 − 500 µg Chl with a Chl a/b ratio of 6 − 7:1. The properties of the core complex are summarized in Table 1.

Biophysical characterization of the core complex

Upon $β$-dodecylmaltoside solubilization and separation on sucrose gradients, the light-harvesting complex became
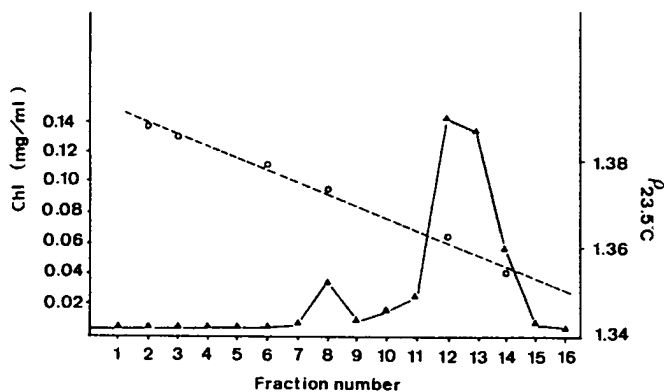
dissociated from the core of PS II. Therefore, on the basis of the total chlorophyll content the specific electron transport activity should increase provided that the functional integrity remains unaffected and light saturation is achieved. In fact, the specific activity of the core complex is markedly smaller than that of the PS II membrane fragments (see Table 1). At the moment we cannot completely rule out a saturation effect. However, a modification of the functional integrity seems to be mainly responsible for the loss of activity. Different levels of distortion have to considered: (a) reduction of the dichloro-indophenol turnover due to a modification of the Q_A site; (b) functional detachment of Q_A leading to a retardation or blockage of electron transfer from pheophytin to Q_A that leads to a drastic reduction of the quantum yield of photochemical charge separation sufficiently stable for dichloroindophenol reduction and (c) impairment of the 1,5-diphenylcarbazide electron donor efficiency. Among these possibilities the PS II acceptor side appears to be the most sensitive part. Effects on herbicide binding after solubilization with detergents indicate the modification of the Q_B site already in PS II membrane fragments [46]. Detergent-induced conformational changes of spectroscopic properties (fluorescence emission at 77 K) and electron transfer rates (diphenylcarbazide → dichloro-indophenol) could also be observed using the intrinsic PS II membrane proteins. The three non-ionic detergents Triton X-100, $β$-N-octylglucoside and $β$-dodecylmaltoside dramatically modified both features with strong dependence on their critical micelle concentrations [47]. The detergent/chlorophyll ratio required for the solubilization of the PS II core complex very likely leads to a more severe structural change of the Q_B site. In addition to that, evidence is provided for an impairment of the Q_A function probably caused by a partial loss and/or modification of the interaction of the D1-D2-cytochrome b_{559} complex with the 47-kDa polypeptide. To our knowledge there is no preparation available so far which is capable of transferring electrons from diphenylcarbazide to dichloroindophenol in the absence of CP47. A recent report by Bassi et al. [48] described the isolation of a complex called Chl$a$P2* which consisted of the 47-kDa, D1 and D2 polypeptides and cytochrome b_{559} with an electron transfer activity half of that determined in this work. This study also confirmed the previous conclusion of Yamagishi et al. [49, 50] that CP43 is not necessary for a functionally active PS II reaction center core. We have also isolated several PS II core subcomplexes with various polypeptide compositions, e.g. CP47, D2, D1 and a 22-kDa pro-
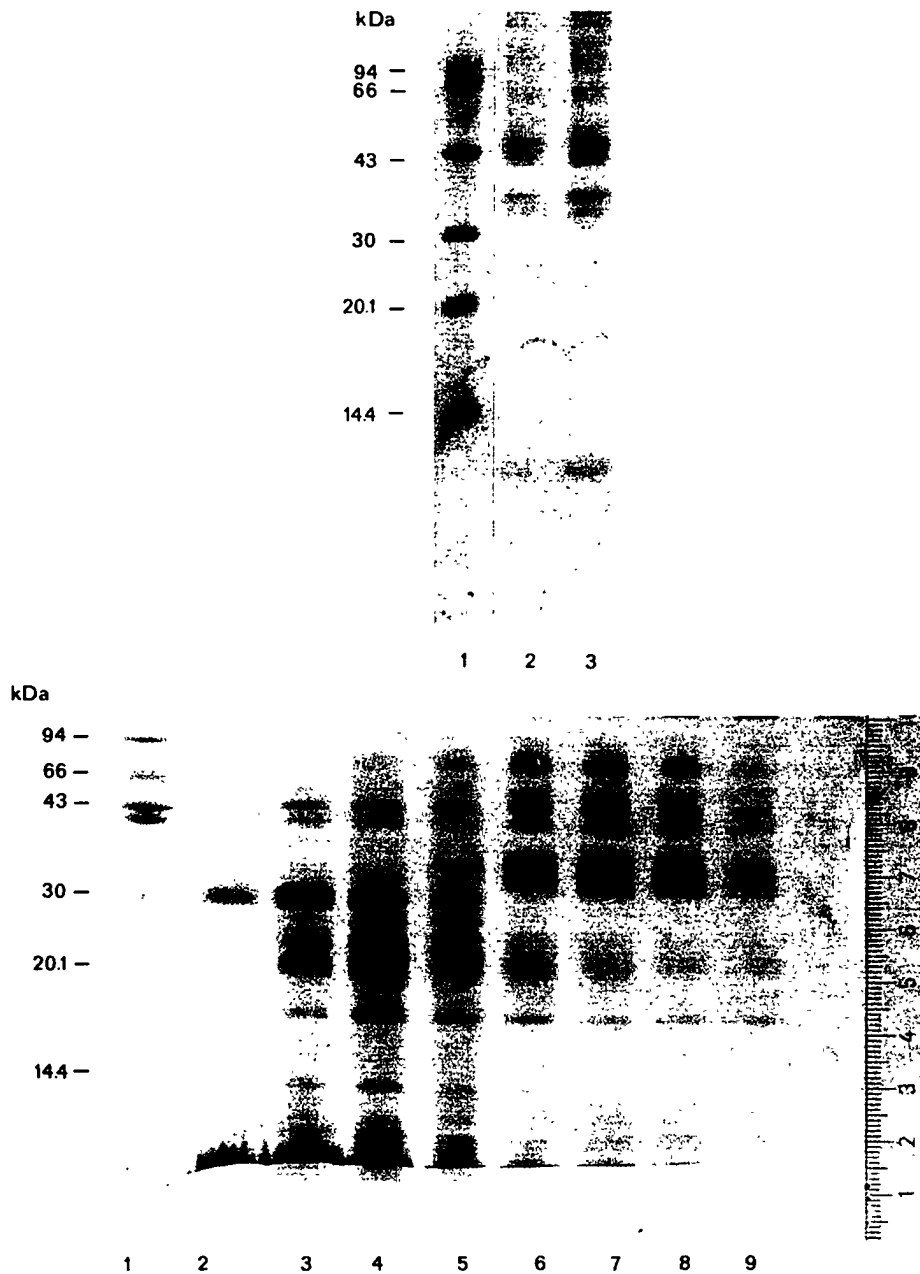
Fig. 3. *SDS/urea/PAGE of PS II intrinsic membrane proteins.* (a) Coomassie-R-250-stained gel: lane 1, molecular mass marker proteins (Pharmacia): lanes 2 and 3, PS II core complex proteins; (b) silver-stained gel: lane 1, molecular mass marker proteins (Pharmacia); lanes 2—5, LHC-containing fractions; lanes 6—9, PS II core complex fractions

tein. Electron transfer activity (diphenylcarbazide → dichloroindophenol) was registered in all cases where CP47 was present (data not shown). This is an indication of a functional role for CP47 in the PS II core complex, besides acting merely as a core antenna, perhaps in stabilizing an appropriate reaction center conformation for charge separation.

The room-temperature absorption spectrum of the core complex showed a maximum at 674 nm due to Chl *a*, but no considerable absorption for Chl *b*. A shoulder near 470 nm indicated the presence of some carotenoids (Fig. 5). The dashed curve of Fig. 5 demonstrates the calculated corresponding second-derivative spectrum. The core complex exhibits one strong band at 670 nm and a shoulder at 679 nm.

Both maxima are in good agreement with the data obtained from low-temperature absorption spectra by van Dorssen et al. [51] for PS II reaction center and isolated CP47. The 77 K fluorescence emission spectrum of the core complex revealed a sharp band at 682 nm with a small shoulder near 690 nm (Fig. 6). After preincubation of the samples with 50 mM guanidinium · HCl, pH 7.5 and 1 mM *o*-phenanthroline we could resolve the superimposed band (data not shown, see also [52, 53]). The calculated second-derivative spectrum resolved two components with maxima of 678 nm and 692 nm (see dashed curve of Fig. 6). The minor component with an emission maximum at 692 nm is attributed to chlorophyll *a* specifically bound to CP47 [51]. The calculated fourth-derivative spectrum (data not shown) suggests that the main band

Table 1. *Characteristics of isolated photosystem II preparations*
Specific activity was measured as the rate of reduction of dichloroindophenol with the numbers in parentheses representing reduced dichloroindophenol generated as a percentage of that generated by membrane fragments. abs., absorbance; fluor., fluorescence; n.d., not determined

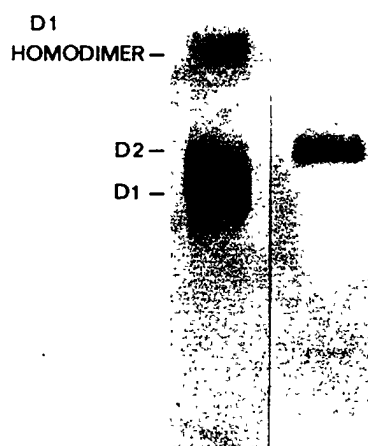| Photosystem II preparation	Specific activity	Ratio of Chl a/b	Chlorophyll yield	Chl a/b λ_{max}		Bands from 2nd derivative abs./fluor.
				abs.	fluor	
	$\mu mol\ mg^{-1}\ h^{-1}$ %		mg	nm		
Membrane fragments	140 – 160 (100)	2.0 – 2.1	3 (starting material)	650 – 652, 672	685, 694, ± 729	n.d.
Intrinsic proteins	60 – 70 (43)	2.0 – 2.1	3	650 – 652, 672	685, 694, ± 729	n.d.
Core complex	30 (20)	6 – 7	0.3 – 0.5	674	682, 692 (shoulder)	670/678 679/692

Fig. 4. *Immunodetection of D1 and D2*. The blotting procedure is described in [40] with modifications as in [41]. Anti-D1 and anti-D2 sera were used in 1/500 dilutions

at 678 nm is actually composed of two species, one peaking at 678 nm and another at 680 nm.

Electron microscopy

The intrinsic PS II membrane proteins isolated by Triton X-100 solubilization from thylakoids followed by depletion of all the hydrophilic polypeptides by a high-salt procedure (Fig. 1) were generally aggregated into double (inside-out) membrane fragments (Fig. 7). The thickness of these sheets amounted to about 6.5 nm, separated by a gap of about 1 nm. If we tried to solubilize the membrane fragments with Triton X-100, under no conditions did we obtain a monodisperse solution. At detergent concentrations above 0.4% (mass/vol.) Triton X-100 we observed a disruption of the double sheets, yielding fragments of a size smaller than expected for the PS II core complex.

In order to obtain monodispersed complexes we changed over to the glycosidic detergent β-dodecylmaltoside. The preparations were negatively stained with uranyl acetate and analyzed by electron microscopy. In the presence of β-dodecylmaltoside the isolated PS II core complexes gave rise to mainly two different projections: a top and a side view. We
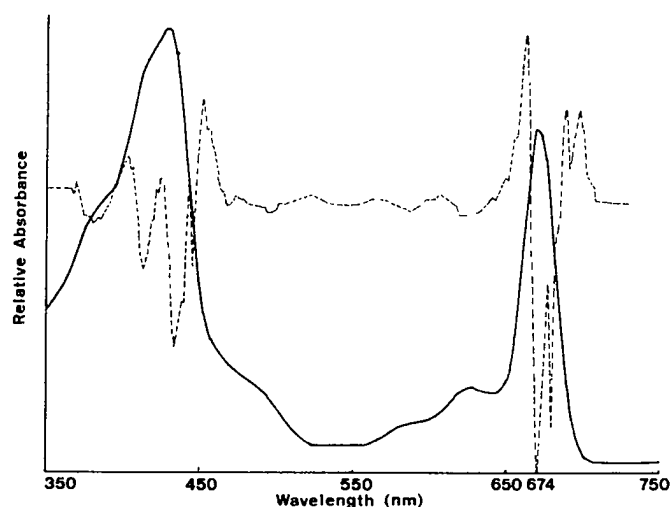

Fig. 5. *Room-temperature absorption spectrum of the PS II core complex isolated by sucrose density gradient centrifugation*. The dashed curve represents the calculated corresponding second derivative spectrum
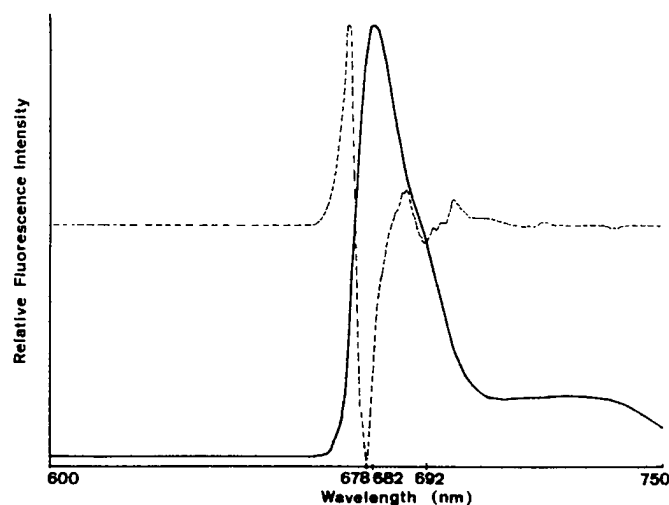

Fig. 6. *Fluorescence emission spectrum at 77 K of the PS II core complex*. Chl concentration: 25 µg/ml. The dashed curve demonstrates the corresponding second-derivative spectrum

Fig. 7. *An electron micrograph of PS II intrinsic membrane proteins isolated in Triton X-100, negatively stained with 1% uranyl acetate*
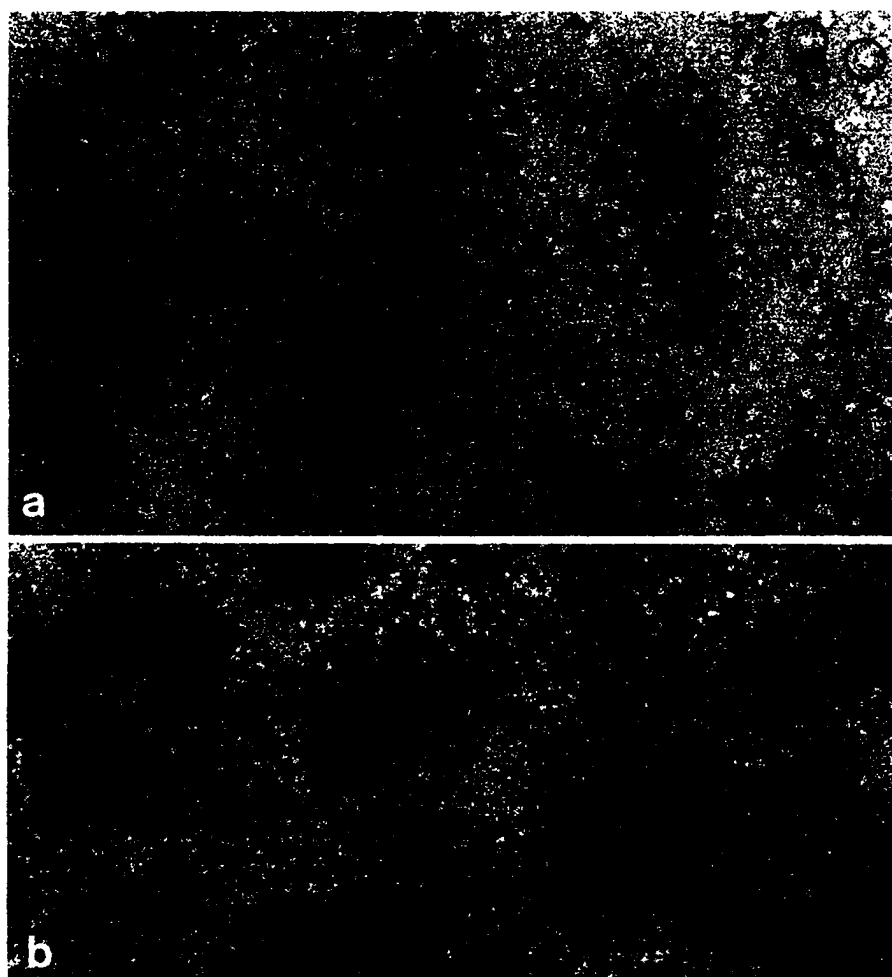

Fig. 8. *Two electron micrographs showing PS II core complexes in different positions on the carbon support.* In (a) many complexes are lying free in top-view projection: in (b) they are mostly in side view position and frequently aggregated into small strings. Specimens were prepared in the presence of 0.015–0.03% β-dodecylmaltoside and stained with 1% uranyl acetate. In (a), some in triangular top views have been circled and side views indicated by arrows
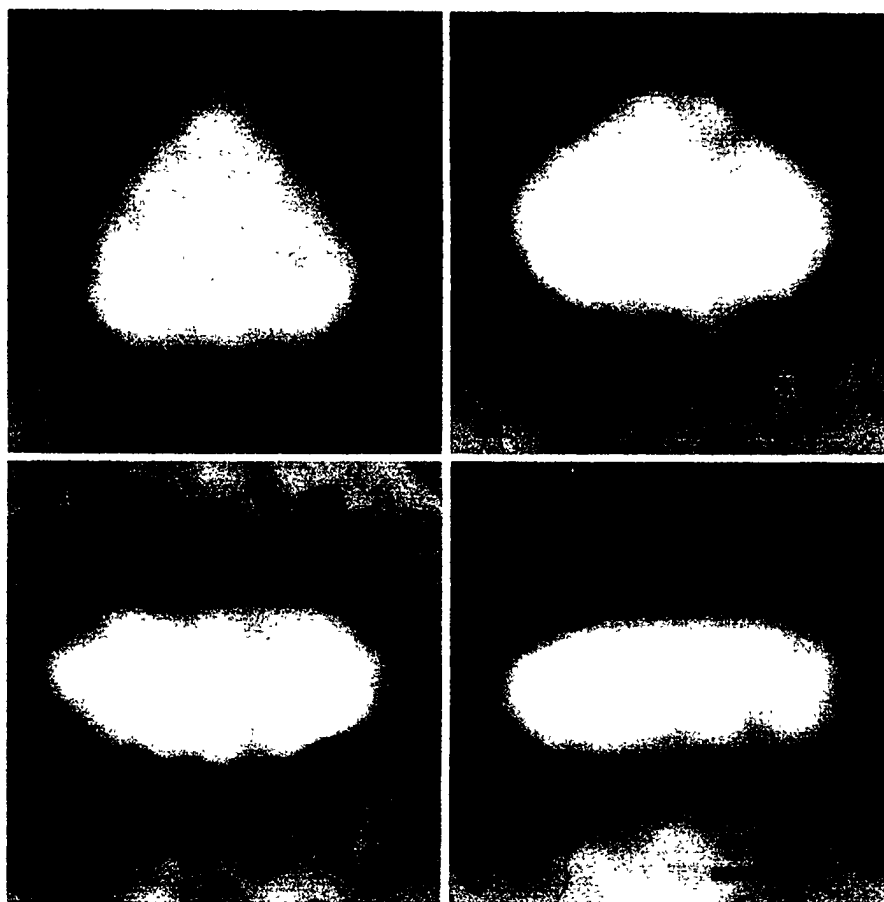

Fig. 9. *Averaged images of projections of PS II core complexes.* (a) Averaged image of 78 projections of the triangular view. (b) Averaged image of 35 projections of the obtuse-triangular view. (c, d) Averaged images of 12 and 24 side-view projections, respectively

frequently observed an aggregation of the complexes with their smallest dimension (the side view) as the repeating unit (Fig. 8B). This aggregation behaviour was also noticed for other photosynthetic membrane proteins [33, 54, 55], as well as the small, but clear, gap between the individual molecules in such aggregates. This typical aggregation behavior is not fully understood, but it is detergent-dependent, since there is an optimum detergent concentration at which this aggregation effect is maximum. Since these aggregates are the result of protein−protein interactions [33, 55], the center-to-center distance of 6.8 nm (n = 32) was an appropiate parameter for estimating the maximum thickness of the PS II core complex. The similar complex from *Synechococcus* sp. has a center-to-center distance of approximately 7.0 nm [56].

On other micrographs single complexes predominated, and the most abundant projection was a top view with a triangular to circular shape (Fig. 8A). A total of 158 of these projections were selected from seven micrographs in arrays of 64 × 64 pixels. The only criterion for selection was that the projections were well stained and not in contact or over-lapping with other projections. They were brought into equivalent position by alignment procedures and averaged by summation. The sum of the best 78 aligned images is shown in Fig. 9A. Although this sum shows no clear internal fea-tures, its outlines are relatively sharp, as should be expected for the average of a homogeneous population of complexes. The dimensions of the triangles are 13 nm × 12.5 nm.

The 146 selected side-view projections differ mainly in width, which is explained by slight tilts on the carbon support.

Most of the projections have a width of 6.5 nm and a length of 15 nm; some show a similar length, but the width is larger (7.2 nm). Other projections were less frequent in these images. Most numerous were characteristic obtuse triangles, inter-mediate in shape between the triangular and the side views. From 64 aligned images the best 35 were summed (Fig. 9B). We interpret them as projections mainly from complexes in a position intermediate between those of Fig. 9A and 9C. The maximum dimensions of the projection of Fig. 9A appear to be slightly smaller than those of Fig. 9B. However, small differences in shape and size have to be interpreted with care since the boundary layer of detergent molecules contributed considerably to the dimensions of the projections. So the discrepancy of about 2 nm between the maximum distances of projections in Fig. 9A vs those in Fig. 9B−D could be partially explained by rearrangements of the detergent mol-ecules around the complexes in the side-view projections.

In summary, we conclude that the PS II core complex of spinach has the shape of a triangular-to-circular flat cylinder with dimensions of 12.5−13 nm in the top view and a maximum height of 6.8 nm. The complex measures about 8−9 nm in diameter, if corrected for the β-dodecylmaltoside boundary layer. These data are in accordance with others [57, 58]. Monomeric PS II particles from the cyanobacterium *Synechococcus* sp. have dimensions of 10.8 nm × 15.4 nm measured for the top-view projection in β-dodecylmaltoside and a height of 7 nm. Comparison of both complexes from spinach and *Synechococcus* shows that they are similar in height but different in their top-view projections. The former

is slightly smaller, but the difference in total volume and mass would be 5 – 10%, which is probably not significant. An important distinction is, however, found in the shape of both complexes. PS II particles of the cyanobacterium are elongated, whereas in preparations of spinach PS II complexes are triangular. We also have no evidence for dimeric complexes as was demonstrated in the case of *Synechococcus* [33, 56, 59]. Perhaps the dimerization of PS II is a characteristic property of organisms with hemi-discoidal extramembrane phycobilisomes as light-harvesting systems, in which the two basal core cylinders are well matched in size to the two PS II monomers [56, 59]. This structural organization seems to be optimized for energy transfer to neighbouring PS II complexes and phycobilisomes. A different situation is imaginable for the structural arrangement of the antenna apparatus and the PS II core complex in higher plants, in which both are intramembrane pigment-protein complexes.

The three-dimensional structure of the light-harvesting chlorophyll *a/b* protein complex from pea and spinach has been recently determined by (cryo)electron microscopy of two-dimensional crystals and image analysis [60 – 62]. This complex exhibited a trimeric structure, composed of three subunits [60 – 62]. There is still some controversy about the polypeptide composition of the monomer, but the LHC reveals in all cases triangular projections which are similar to, but slightly smaller than those of the PS II core complex reported here. From gel electrophoretic and spectroscopic analyses we are assured that our preparation is free from LHC polypeptides (Figs 3A, B and 5). Possibly, a triangular structure of the PS II core complex is required for realization of an optimum assembly with the LHC II units (1/3 trimer) or LHC trimers. This would also provide for an economical organization of antenna complex and reaction center core, in terms of efficient excitation energy transfer [63].

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