ISOLATION OF THE PHOTOSYSTEM II REACTION CENTER COMPLEX FROM BARLEY. CHARACTERIZATION BY CIRCULAR DICHROISM SPECTROSCOPY AND AMINO ACID SEQUENCING

by

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A photosystem II (PSII) reaction center complex containing Chl_a-protein 2, Chl_a-protein 3, cytochrome b₅₅₉, the herbicide-binding polypeptide and the 33 kD polypeptide from the oxygen-evolving site of PSII was isolated by sucrose gradient centrifugation of the dodecyl- β ,D-maltoside extract of grana membranes highly enriched in PSII. The reaction center complex contained ca 19 lipid molecules per reaction center and sedimented slightly faster than catalase. It mediated light-induced, DCMU-sensitive electron transport from 1,5-diphenylcarbazide to dichlorophenolindophenol.

The N-terminal amino acid sequence of a proteolytic fragment of Chl_a-protein 2 was determined. Eighteen out of 20 amino acid residues were identical with the deduced amino acid sequence of the 51 kD Chl_a-apoprotein from spinach.

In contrast to grana membranes, which had a 77 K fluorescence emission maximum at 685 nm with a pronounced shoulder at 695 nm, the PSII reaction center complex had a single peak at 685 nm. Circular dichroism measurements in the visible range showed that the pigments associated with the isolated PSII reaction center complex and the light-harvesting complex (LHC) were highly oriented. The secondary structure of the PSII reaction center complex and LHC was investigated by circular dichroism measurements in the ultraviolet range. Both had a high α -helix content, suggesting that the chlorophyll molecules may be oriented between transmembrane helices in a similar way as in bacterial reaction centers and light harvesting systems.

1. INTRODUCTION

Over the last seven years many different PSII preparations with varying degrees of complexity have been described. Preparations of grana membranes highly enriched in PSII by phase partitioning (1, 20) or Triton-treatment (7, 30)

made it possible to study the function of PSII in a system which was close to the *in vivo* situation and to investigate the lateral distribution of proteins and lipids in the thylakoid membrane. Additionally, grana membranes were fractionated by extraction with various detergents fol-

Abbreviations: Chl = chlorophyll; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP = 2,6-dichlorophenolindophenol; DPC = 1,5-diphenylcarbazide; HEPES = N-(2-hydroxyethyl)-piperazine-N'-2-ethane sulfonic acid; LHC = light harvesting complex; LHCII = LHC of photosystem II; PAGE = polyacrylamide gel electrophoresis; PS = photosystem; PTH = phenylthiohydantoin; SDS = sodium dodecyl sulfate; tricine = N-(tris(hydroxymethyl)methyl)glycine; Tris = tris-(hydroxymethyl)aminomethane.

lowed by column chromatography, sucrose gradient centrifugation or isoelectric focussing (17, 38, 39, 46, 49). Unfortunately many of these procedures were rather time-consuming or gave only low yield. With one exception (17), the lipid content of the preparations was not investigated.

The genes of proteins involved in photosynthetic electron flow are generally highly conserved as exemplified by the 32 kD herbicide-binding polypeptide. The nucleotide sequence of this gene has been determined in a variety of organisms, showing very few differences between cyanobacteria (13), *Chlamydomonas* (15) and higher plants (50). The sequence of the D2 membrane polypeptide from PSII is also conserved (3, 22, 37). The few differences occurring in the sequences of these polypeptides suggest that their tertiary structure and the architecture of the PSII core complex with which they interact may be equally conserved.

The molecular architecture of bacterial reaction center complexes has been the subject of numerous investigations. Recently the reaction center complex from a photosynthetic bacterium has been crystallized and its structure analysed at 3 Å resolution, displaying transmembrane α -helices and the organization of the pigments (14, 28). The structure of PSI and LHCII from higher plants has been studied by circular and linear dichroism spectroscopy, indicating the existence of transmembrane α -helices slightly tilted relative to the membrane normal and an extremely low content of β -structure (9, 31, 32). In contrast, very little is known about the structure of the PSII reaction center complex in higher plants.

We describe here the isolation of a functional PSII reaction center complex containing 5 polypeptides and ca 19 lipid molecules by a fast and simple method. The secondary structure of this reaction center complex and the organization of its pigments were investigated by circular dichroism measurements and compared with the bacterial system.

2. MATERIALS AND METHODS

2.1. Biological preparations

Wild type barley seedlings (*Hordeum vulgare* cv Svalöf's Bonus) were grown for 7 days in

vermiculite under continuous illumination (1700 lux). The mutant *chlorina-f2* was grown under the same conditions. Mutant and wild type thylakoids were prepared for SDS-PAGE as described in (25). Grana membranes were prepared using a modification of the Triton X-100 method of BERTHOLD et al. (7) as described in (30).

2.2. Isolation of the PSII reaction center complex

For the isolation of the PSII reaction center complex grana membranes were solubilized by dodecyl- β ,D-maltoside and fractionated on sucrose gradients in a similar way as described in (47). The membranes were diluted to a final concentration of 1 mg $\cdot$ ml⁻¹ chlorophyll in a buffer containing 20 mM-HEPES, 200 mM-NaCl, 1 mM-EDTA, pH 8.0. Ten mg dodecyl- β ,D-maltoside per mg chlorophyll were added and the sample was incubated for 10 min at room temperature. All further steps were performed at 4 °C. The sample was centrifuged for 10 min at 16.000 g to pellet insoluble material. The supernatant was applied to gradients containing 10-30% sucrose, 0.02% Triton X-100, 10 mM-Tricine, pH 8.0. After 24 h centrifugation at 83.000 g fractions were collected from the top. Material which was not used immediately was frozen rapidly in liquid nitrogen and stored at -30 °C.

2.3. Spectroscopic measurements

Absorption spectra were recorded using an Aminco DW2a spectrophotometer. The concentration of the samples was adjusted to an absorbance of ca 0.3 at 670 nm.

Fluorescence emission spectra at liquid nitrogen temperature were measured using a fiber optic system (Applied Photophysics, London, England) as described in (41).

Room temperature circular dichroism spectra were obtained on a Roussel-Jouen Dichrograph III. The length of the optical cell was 1 cm, enabling us to take spectra of dilute samples.

For measurements in the ultraviolet range (200-260 nm) sucrose was removed from the gradient fractions by gel-filtration over an AcA

34 column equilibrated in 0.02% Triton X-100, 10 mM-Tricine, pH 8.0.

2.4. Electron transport measurements and determination of cytochrome content

PSII activity was determined as DCMU-sensitive photoreduction of DCPIP by following the rate of decrease of the absorbance at 575 nm using 1,5-diphenylcarbazide as electron donor.

The cytochrome content was determined from reduced minus oxidized difference spectra as described in (30).

2.5. Lipid analysis

Ten volumes of chloroform : methanol (1:1 v/v) were added to each of the samples in a teflon lined screw cap tube. After vortexing vigorously for 3 min, 2 volumes of 25% NaCl were added and the chloroform phase pipetted off. Two additional extractions with 2 volumes of chloroform were made. The combined chloroform extracts were dried over anhydrous Na_2SO_4 , passed through a cotton plug in a Pasteur pipette into a clean screw cap tube and taken to dryness under a stream of nitrogen. After the addition of 206 nmoles of pentadecanoic acid the lipids were transesterified using 3 ml of 14% BF_3 in methanol and heating for 20 min at 80 °C.

Cooling and dilution with 2 ml water plus 2.5 ml 25% NaCl were followed by 3 extractions of the solution with 2-3 ml hexane. After the removal of hexane under a stream of argon at 20 °C or less, aliquots in hexane were analyzed by gas chromatography using a 10% Silar 10C column (5) in a Hewlett Packard Model 5840A instrument. The column temperature was held at 150 °C for 5 min before heating at 3° · min⁻¹ to 200 °C. Injector and detector temperatures were 220 and 350 °C, respectively. To allow for quantitation, calibration was carried out as described in (35) with methyl pentadecanoate, methyl palmitate and methyl stearate from Applied Science (Oud-Beijerland, Holland). The flame ionization detector response factors given in (42) were used.

2.6. Electrophoresis

One mm thick SDS-polyacrylamide gels containing 12.5 or 15% polyacrylamide were used

for both analytical and preparative purposes. The gels were run at 4 °C using buffer system II (25). The samples were solubilized using 10 mg dodecyl- β ,D-maltoside per mg Chl (final concentration 1%) and the SDS concentration in the gels was lowered to 0.03% to minimize pigment loss. Proteins were visualized by staining with Coomassie Brilliant Blue R250 or by the alkaline silver stain described in (48).

For the isolation of Chl_a -protein 2 and Chl_a -protein 3 by preparative SDS-PAGE *chlorina-f2* thylakoids were used as the starting material to avoid contamination by oligomers of LHCII. The bands containing Chl_a -protein 2 and Chl_a -protein 3 were cut out from unstained gels. The gel slices were ground using a glass rod in a Nunc Minisorp test tube and incubated for 12 h at 4 °C with five volumes of distilled water. This extraction step was repeated with 0.05% SDS in distilled water. Polyacrylamide fragments were removed by centrifugation. The supernatants were lyophilized and redissolved in a small volume of distilled water.

Proteolytic digestion with *Staphylococcus aureus* protease V8 (Miles Laboratories) was performed during SDS-PAGE as described in (11). Purified Chl_a -protein 2 was applied to a 15% polyacrylamide gel, together with increasing amounts of protease (0.1-10% w/w). These gels were run without cooling. They were stained with Coomassie Brilliant Blue R250. Bands containing 5-20 μg material were excised and the peptides were eluted with 70% formic acid as described in (40) or with 50% acetic acid. Formic acid (70%) is a better solvent for peptides than acetic acid (50%), but has the disadvantage that it tends to react with their free N-termini. The eluted material was dried under vacuum. The peptides were redissolved in 60 μl 70% formic acid and separated from buffer components and other low molecular weight compounds by gel filtration on a 30 cm long Sephadex G50 column (column volume 2 ml) equilibrated in the same solvent. The peptide-containing fractions were lyophilized after the addition of 40 μl 0.1% SDS. They were redissolved in 40 μl distilled water and applied to a gas-phase protein sequencer (Applied Biosystems 470 A). PTH-amino acids were identified by HPLC.

Figure 1. Polypeptide composition of the preparations as revealed by SDS-PAGE. Gels containing 12.5% polyacrylamide were run at 4 °C and stained with Coomassie Brilliant Blue. A: thylakoids. B: dodecyl-β,D-maltoside extract of grana membranes. C-F: gradient fractions. C: salt-releasable polypeptides. D: LHCII. E: aliquot from the region between the two green bands. F: PSII reaction center complex.

2.7. Other methods

The chlorophyll concentration was measured in 80% acetone according to the method of ARNON (4). Protein was quantitated as described by LOWRY et al. (24), using bovine serum albumin as a standard. The amino acid composition of the proteins was determined after 24 h hydrolysis at 108 °C in 6 N-HCl in evacuated, sealed tubes.

3. RESULTS

3.1. Isolation of the PSII reaction center complex

A functional PSII reaction center complex was prepared by sucrose gradient centrifugation of dodecyl-β,D-maltoside extracts of grana membranes highly enriched in PSII. The polypeptide composition of the different fractions is shown in Figure 1. The colourless fraction at the

Table I.**Characteristics and yield of fractions obtained during the isolation of the PSII reaction center complex.**

Fraction	chlorophyll (mg)	protein (mg)	Chl <i>a/b</i>	protein/ Chl	μmoles DCPIP/ h · mg Chl
Thylakoids	215	2580	2.9	12	8.7
Grana membranes	63	630	2.3	10	nd
Dodecyl-maltoside extract	50	500	2.3	10	44
LHCII	44.5	405	1.6	9.1	nd
PSII reaction center complex	5.5	42	9.3	8.7	36

nd: not determined

top of the gradient contained equal amounts of the two salt-releasable polypeptides with apparent molecular weights 24 kD and 13.5 kD plus traces of a 33 kD polypeptide. The association of these polypeptides with the oxygen-evolving system and their release by high salt or alkaline Tris has been described by several authors (1, 30, 46, 49).

The upper deep green band containing LHCII had a Chl *a/b* ratio of 1.6 (Table I), while that of the lower green band containing the PSII reaction center complex ranged from 8 to 10. The PSII reaction center complex contained Chl_a-protein 2, Chl_a-protein 3, cytochrome *b*₅₅₉, the herbicide-binding polypeptide and at least one more polypeptide in the 33 kD region (Figure 1). The 10 kD band may represent the apoprotein of cytochrome *b*₅₅₉. The faint band comigrating with Chl_a-protein 1 was probably a protein aggregate since the extract was strongly depleted in PSI. The herbicide-binding polypeptide did not stain with Coomassie Brilliant Blue or silver. Its presence was deduced from the observation that electron transport through the PSII reaction center complex was fully sensitive to DCMU. The 33 kD polypeptide stained well with Coomassie Brilliant Blue. When its amino acid composition was determined (Table III), it was found to contain lysine, indicating that this 33 kD polypeptide is not related to the quinone-binding polypeptide of similar size since the latter is completely deficient in lysine (50). The amino acid compositions of the 33 kD polypeptide associated with the PSII reaction center

complex and of the 32 kD polypeptide from the oxygen-evolving system described in (30) are similar, indicating that they are one and the same polypeptide. Except for the low potential form of cytochrome *b*₅₅₉, no other cytochromes were detected by reduced minus oxidized difference spectra. During sucrose gradient centrifugation the PSII reaction center complex sedimented as a sharp band and slightly faster than catalase (250 kD), indicating that it was composed of particles homogeneous in size and not of membrane fragments.

Measurements of light-induced electron transport from DPC to DCPIP are summarized in Table I. The PSII reaction center complex showed higher rates of electron transport than thylakoids on a Chl basis because of the removal of Chl associated with LHC and PSI. The isolated PSII reaction center complex was fully sensitive to DCMU.

Absorption spectra of the PSII reaction center complex and LHCII are shown in Figure 2. The PSII reaction center complex had an absorption maximum at 671 nm. In the case of LHCII this peak was shifted to 670 nm and had a shoulder at 652 nm due to Chl *b*. The absorption spectrum of LHCII had an absorption maximum at 467 nm reflecting its high content of Chl *b* and carotenoids. The spectrum of the PSII reaction center complex has a shoulder in this region, showing that it does contain small amounts of carotenoids.

The 77 K fluorescence emission spectra of grana membranes, the PSII reaction center com-

Figure 2. Absorbance spectra of the PSII reaction center complex (bottom) and LHCII (top). The samples were adjusted to an absorbance of ca 0.3 at 670 nm. The spectrum of LHC is shifted upwards relative to that of the PSII reaction center complex to allow details of both spectra to be seen clearly.

Figure 3. Low temperature (77 K) fluorescence emission spectra of grana membranes, the PSII reaction center complex and LHCII.

plex and LHCII are shown in Figure 3. Grana membranes had a fluorescence emission maximum at 685 nm with a pronounced shoulder at 695 nm. There was very little fluorescence at longer wavelengths, where pigments associated with PSI emit (41). The PSII reaction center

complex had a fluorescence maximum at 685 nm without a shoulder at 695 nm. The fluorescence maximum of LHCII was at 680 nm.

3.2. Lipid content of grana membranes and the PSII reaction center complex

Lipids were quantitated as fatty acid methyl esters after hydrolysis with BF_3 . The results are

Table II.
Lipid analysis.

Fraction	$\mu\text{mole lipid}/$ $\text{mg Chl } a$	$\text{nmole lipid}/$ mg protein	Fatty acid distribution (mole %)					
			16:0	16:1	18:0	18:1	18:2	18:3 ^{a)}
Thylakoids	5.5	389	9.5	1.8	0.4	0.9	5.8	81.7
	5.1	360	9.3	1.9	0.4	1.0	5.7	81.8
	5.9	416	8.3	1.7	0.3	0.8	5.5	83.3
Grana membranes	1.4	76	20.6	3.7	2.4	2.4	9.0	61.9
	1.3	75	20.6	3.7	2.5	2.3	9.2	61.7
	1.2	67	20.9	3.7	2.6	2.6	9.2	61.1
	0.9	61	22.4	3.6	3.5	4.3	10.6	55.5
PSII reaction center complex	0.6	50	23.6	6.4	3.2	4.7	7.2	54.9
	1.0	80	21.5	5.5	4.1	5.3	9.7	54.0
	0.6	56	24.2	5.1	1.7	5.9	12.6	50.5

a) 16:0 = palmitic acid; 16:1 = hexadecanoic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 18:3 = α -linoleic acid

presented in Table II. Thylakoids contained 5.5 μmole acyl lipids per mg Chl *a*, or 390 nmole lipid per mg protein. Grana membranes prepared by Triton-treatment had only 20% of the lipid content of thylakoids relative to Chl and protein. There were 1.24 μmole lipids per mg Chl *a* or 72 nmole lipid per mg protein. The PSII reaction center complex contained an average of 0.7 μmole lipid per mg Chl *a* or 62 nmole lipid per mg protein, indicating that there was a core of tightly bound lipids which were not removed by detergent extraction and sucrose gradient centrifugation. α -Linoleic acid ($\text{C}_{18:3}$) was by far the most abundant fatty acid in thylakoids. During purification the amount of α -linoleic acid (mole %) decreased from 82% in thylakoids to 55% in the PSII reaction center complex and the amount of palmitic acid ($\text{C}_{16:0}$) increased from 9 to 22%.

3.3. Amino acid composition and sequence analysis

In order to avoid contamination by oligomers of LHCII the Chl_a-proteins 2 and 3 were prepared from the mutant *chlorina-f2* as described in 2.6. As the N-terminus of Chl_a-protein 2 was apparently blocked, the intact protein could not be used for amino acid sequence analysis. Instead, after partial proteolytic digestion of Chl_a-protein 2 with *Staphylococcus aureus* protease V8, three of the peptides with apparent molecular weights 11.5, 9.9 and 6.6 kD were isolated by preparative SDS-PAGE. All three peptides had the same N-terminal amino acid sequence (Figure 4), indicating that the 9.9 and the 6.6 kD peptides were derived from the 11.5 kD peptide by further cleavage in the C-terminal part of the molecule. The repetitive yield during sequencing was 88%. Eighteen out of 20 amino acid residues in this sequence are identical with a part of the sequence deduced from the nucleotide sequence of the gene for the 51 kD Chl_a-apoprotein from spinach, starting with residue 401 (29). At position 408 a glutamic acid residue has been replaced by glycine. The other change is conservative, asparagine in position 410 instead of serine. Moreover, the deduced amino acid sequence of the 51 kD Chl_a-apoprotein had a glutamic acid residue directly in front of the N-ter-

401
Spinach : Glu Phe Tyr Gly Gly Glu Leu Asn Glu Val Ser
Barley : Phe Tyr Gly Gly Glu Leu Asn Gly Val Asn
411
Tyr Ser Asp Pro Ala Thr Val Lys Lys Tyr
Tyr Ser Asp Pro Ala Thr Val Lys Lys Tyr

Figure 4. Sequence homology between the N-terminal amino acids of the 9.9 kD proteolytic peptide of ChL₂-protein 2 from barley and a portion of the deduced amino acid sequence of the 51 kD ChL₂-apo-protein from spinach starting at residue 401.

minus of the analyzed peptides. This was expected because *Staphylococcus aureus* protease V8 cuts preferentially after glutamic acid residues. It also cuts after aspartic acid residues but with lower efficiency. The apparent molecular weights of the three proteolytic peptides were consistent with the positions of glutamic and aspartic acid residues in the sequence of the spinach 51 kD Chl_a-apoprotein.

Table III lists the amino acid composition of Chl_a-protein 2, its 9.9 kD proteolytic fragment and of Chl_a-protein 3. The amino acid compositions of the 51 kD Chl_a-apoprotein and of the 44 kD Chl_a-apoprotein from spinach which were deduced from the nucleotide sequence of their respective genes (3, 29) are shown for comparison. The amino acid composition of Chl_a-protein 2 was similar to that of the 51 kD Chl_a-apoprotein from spinach and the amino acid composition of Chl_a-protein 3 resembles that of the 44 kD Chl_a-apoprotein, suggesting that the proteins from barley and spinach are closely related.

The amino acid composition of Chl_a-protein 2 and Chl_a-protein 3 shows that both proteins are highly hydrophobic. They have 47.2 and 44.7% nonpolar amino acids respectively. A ratio of polar to apolar amino acids close to one is typical for integral membrane proteins whereas soluble proteins usually have a ratio of polar to apolar residues close to two (10, 18, 19).

3.4. Circular dichroism studies

Ultraviolet circular dichroism spectra of gradient fractions containing the PSII reaction

Table III.
Comparison of amino acid compositions (mole %) of purified barley and spinach thylakoid proteins.

	Chl _a - ¹⁾ protein 2	9.9kD ¹⁾ fragment	51 kD Chl _a - ²⁾ apoprotein	Chl _a - ¹⁾ protein 3	44 kD Chl _a - ³⁾ apoprotein	33 kD polypeptide	¹¹ 32 kD ⁴⁾ polypeptide
Asx	8.6	9.9	6.7	8.4	5.9	10.3	8.5
Thr	5.6	5.9	4.9	5.2	5.3	5.8	8.2
Ser	6.9	6.5	6.7	7.5	5.7	6.6	6.4
Glx	7.8	9.8	6.9	8.9	6.3	10.6	11.9
Pro	5.9	4.0	5.3	5.5	5.3	5.0	6.1
Gly	13.8	12.9	11.8	14.6	12.5	13.9	10.9
Ala	9.2	8.6	7.7	9.5	8.5	8.8	6.8
Val	7.2	6.6	7.9	6.5	6.8	5.9	6.1
Met	1.3	0.9	2.2	trace	1.7	2.4	1.8
Ile	4.7	3.6	5.9	4.0	5.5	4.6	3.6
Leu	11.2	8.6	7.7	12.0	12.1	8.0	8.2
Tyr	2.0	2.8	3.4	2.8	3.2	2.3	3.4
Phe	7.7	8.9	8.1	7.4	7.0	3.9	6.2
His	nd	1.0	2.8	nd	2.8	0.5	1.0
Lys	3.4	4.2	2.6	4.1	2.8	8.6	8.3
Arg	4.7	5.9	5.7	3.4	4.4	2.8	2.9
Cys	nd	nd	0.6	nd	0.9	nd	nd
Trp	nd	nd	3.4	nd	3.6	nd	nd
% nonpolar residues	47.2	41.2	43.1	44.7	45.8	38.6	38.8
polarity index	0.97	1.29	0.90	1.06	0.82	1.5	1.4

The amino acid composition was determined after 24 h hydrolysis.

1) This work. 2) Deduced from the nucleotide sequence in (29). 3) Deduced from the nucleotide sequence in (3). 4) From (30).
nd: not determined

Figure 5. Ultraviolet circular dichroism spectra of the PSII reaction center complex and LHCII.

center complex and LHCII are shown in Figure 5. The shape of the curves indicates that both complexes have a high content of α -helix. A broad trough with minima at 222 nm and 208 nm and an intersection with the baseline close to 200 nm is typical for α -helical polypeptides. β -pleated sheet gives rise to spectra with a narrow, single trough with a minimum at 217 nm. In the visible range the circular dichroism spectra of the two preparations differed markedly as shown in Figure 6. The split peak underlies the red absorption maximum of the PSII reaction center complex with a negative component at 686 nm and a positive component at 673 nm. A small double trough in the region between 440 and 500 nm indicates that a few highly oriented carotenoid molecules are associated with the PSII reaction center complex. The positive circular dichroism signal at 409 nm is due to Chl *a*. The spectrum of LHC had two troughs in the red with minima at 689 and 657 nm. The former signal can be attributed to Chl *a*, the latter to Chl *b*. The strong circular dichroism signals between 440 and 500 nm indicates the existence of highly oriented species

of Chl *b* and carotenoids. Further investigations are needed to assign single peaks to discrete components as well as to explain the origin of the opposite signals at 438 nm of the two preparations. Upon denaturation by 4 M-guanidium chloride the circular dichroism signals of the PSII reaction center complex and of LHCII disappeared, showing that the spectra observed were not due to the intrinsic dichroism of the pigments, but to their organization in pigment-protein complexes. The intrinsic dichroism signals of the pigments were too weak to be detected at the concentrations used.

4. DISCUSSION

A PSII reaction center complex from barley was isolated by a one step procedure using sucrose gradient centrifugation. Inclusion of 200 mM-NaCl in the solubilization medium made it possible to omit washing of the membranes with high salt as a separate step. The Triton concentration in the gradients was lower than in (47) which is an advantage as high concentrations of this detergent were found to denature PSII. Dodecyl- β ,D-maltoside extracts of grana membranes highly enriched in PSII were separated into the PSII reaction center complex, LHCII and salt-releasable polypeptides. In comparison with other methods for the preparation of PSII reaction center particles (17, 38, 39, 46, 49) the one described here has the advantages of being fast, simple and giving high yield.

The PSII reaction center complex was functional since it mediated light-induced, DCMU-sensitive electron transport from DPC to DC-PIP. It contained at least five polypeptides: Chl_a-protein 2, Chl_a-protein 3, the herbicide-binding polypeptide, cytochrome *b*₅₅₉ and a 33 kD polypeptide. Most of the PSII reaction center complexes described retain a 33 kD polypeptide although they are prepared by quite different methods (17, 46, 49). Only when drastic procedures such as washing with alkaline Tris are used is it absent (47). The amino acid composition of the presently isolated 33 kD polypeptide was essentially identical with that of the 33 kD polypeptide from the oxygen evolving system described in (30). Very minor quantities of this 33 kD polypeptide were found together with

Figure 6. Visible range circular dichroism spectra of the PSII reaction center complex (A) and LHC (B).

the salt-releasable 24 and 13.5 kD polypeptides at the top of the gradient, confirming that it interacts directly with the PSII reaction center complex and that it is tightly bound. The isolated polypeptides have no detected enzymatic activity, but they are assumed to stabilize manganese and other ions in the oxygen evolving system (2, 16, 49).

Very hydrophobic proteins, such as the Fo part of the membrane-bound ATPase, interact strongly with lipids and indeed lose activity when the lipids are completely removed (12). Since the PSII reaction center complex is com-

posed of hydrophobic proteins, we analyzed the lipid content of the preparations. Grana membranes obtained by Triton-treatment contained half as much lipid as those obtained by phase partitioning as described in (20, 21). This was reasonable as no detergent is used in the phase-partitioning procedure. Despite this difference in lipid content both types of preparations appear as membranes in the electron microscope (30). The remaining lipids are sufficient to maintain the functional integrity of Triton-treated grana membranes, since MØLLER and HØJ (30) were able to measure the formation of proton

gradients. The isolated PSII reaction center complex contained on the average 0.7 μ moles lipid per mg Chl *a* or 62 nmoles lipid per mg protein. This corresponds to a 11% reduction relative to Triton-treated grana membranes on a protein basis, and the preparation contained ca 19 lipid molecules per reaction center. These lipids were tightly bound and could not be removed without denaturation of the PSII reaction center complex. The lipids associated with the presently isolated PSII reaction center complex were enriched in palmitic acid ($C_{16:0}$) and depleted in α -linoleic acid ($C_{18:3}$) with respect to the starting thylakoids. This observation is interesting in connection with the fatty acid composition determined for individual lipid classes from phase-partitioned grana membranes (21). That is, galactolipids had a very high content of α -linoleic acid in contrast to the other lipid classes. This suggests that the decrease in lipids in the PSII reaction center complex is due to the preferential removal of galactolipids, as opposed to sulfolipids and phospholipids. This is in agreement with the finding that the enzymatic hydrolysis of phospholipids inhibits specifically PSII (23). All this indicates that lipids are an essential part of the PSII reaction center complex and that their composition is different from the overall lipid composition of grana membranes. The presence of these lipids makes it difficult, if not impossible, to obtain three-dimensional crystals of the functional PSII reaction center complex from barley and to analyze its structure and the organization of its pigments by X-ray diffraction, as has been described for a bacterial reaction center complex (14).

Spectroscopic procedures have been used to monitor the structural integrity of Chl-protein complexes and for their characterization. Grana membranes have a low temperature fluorescence emission maximum at 685 nm with a shoulder at 695 nm. This fluorescence at 695 nm has been attributed to the reaction center of PSII itself (8, 33, 34, 45). The fluorescence at 685 nm was thought to arise from Chl molecules with a light-harvesting function. The presently isolated reaction center complex had a fluorescence emission maximum at 685 nm without a shoulder at 695 nm. Since this prep-

aration was active, it is concluded that the fluorescence at 685 nm has contributions both from the reaction center itself and from antenna Chl molecules within the same complex. The fluorescence at 695 nm may reflect the aggregation state of the Chl-protein complex. Aggregation or treatment with cupric phenanthroline can shift the fluorescence emission to longer wavelengths (33, 34, 45). Cupric phenanthroline catalyzes protein crosslinking via disulfide bridges (43, 44) and may thereby cause aggregation.

Circular dichroism studies in the visible range confirmed that the pigment molecules associated with the PSII reaction center complex and with LHCII retained a high degree of organization during the purification procedure. The circular dichroism of the isolated, functional PSII reaction center complex from higher plants has not been previously investigated. It has a split peak underlying the red absorption maximum of Chl_{*a*}, a feature also found in the reaction center of PSI (32) and in bacterial reaction centers (26), but not in LHC. This is thought to be due to exciton interaction between two closely associated Chl molecules (26), and the existence of such a pair in a bacterial reaction center complex has been demonstrated by crystallographic analysis (14). The spectrum of the intact PSII reaction center complex was simpler than that of Chl_{*a*}-protein 2 and Chl_{*a*}-protein 3 isolated by preparative SDS-PAGE (6), perhaps due to contamination of the latter by LHC I-730.

Ultraviolet circular dichroism spectra of the presently isolated PSII reaction center complex and LHCII showed that their secondary structure had a high content of α -helix. Similar results have been described for isolated PSI particles and LHC (9, 31, 32), indicating up to 50% α -helix and maximally 10% β -pleated sheet. Our results are in agreement with the prediction of a high content of α -helix for thylakoid membrane proteins based on the circular dichroism of sonicated thylakoid membranes (36). A high content of α -helix seems to be typical for Chl-proteins, also for those from photosynthetic bacteria. So far, only one exception is known, a water-soluble Chl_{*a*}-protein from a marine alga which an extremely high content of β -pleated

sheet (27). The similarity of the secondary structure of most Chl-proteins suggests that also the mode of pigment organization may be similar and that the Chl molecules in the PSII reaction center complex may be arranged between transmembrane helices with the plane of the pigments more or less vertical to the plane of the membrane analogous to the structure of a bacterial reaction center complex (14).

The number of known chloroplast coded gene sequences and thus of deduced amino acid sequences is increasing faster than the number of directly determined chloroplast protein amino acid sequences. As a consequence indirect approaches have to be used to assign functions to the deduced amino acid sequences. The gene for the 51 kD Chl_a-apoprotein in spinach was identified using an antibody raised in rabbits against the corresponding Chlamydomonas protein (47). We are now able to confirm this assignment. We have obtained a sequence of 20 amino acids after partial proteolytic digestion of Chl_a-protein 2. This sequence is identical in 18 out of 20 positions to that deduced from a portion of the specified spinach nucleotide sequence (29). Moreover the size of the isolated peptides was in agreement with the positions of glutamic and aspartic acid residues predicted from the gene sequence. That the barley sequence is so similar to the deduced spinach sequence and that an antibody raised against Chlamydomonas will precipitate an in vitro translated spinach protein supports our proposal in the introduction that Chl_a-protein 2 is conserved to the same extent as the D2 and the 32 kD herbicide-binding polypeptide (3, 13, 15, 22, 37, 50). In this connection it should be mentioned that the isolated peptides were derived from a relatively hydrophilic part of the molecule as judged from the deduced spinach protein sequence. This part of the polypeptide chain is probably not buried in the membrane and therefore not involved in pigment-binding. The function of this part of the protein is unknown, but the high degree of conservation suggests an important role.

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