POLYPEPTIDE COMPOSITION OF AN OXYGEN EVOLVING PHOTOSYSTEM II VESICLE FROM SPINACH CHLOROPLASTS

by

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An oxygen evolving photosystem II vesicle has been isolated from spinach chloroplasts by the technique of phase-partitioning. The rate of oxygen evolution was comparable to that of unfractionated thylakoids. The vesicle released protons into the ambient medium upon illumination. It emitted fluorescence strongly at 695 nm whereas the fluorescence at 729 nm was weak. The vesicle was enriched in cytochrome *b*-559HP and contained little if any cytochrome *b*-563. The photosystem II vesicle contained four chlorophyll-proteins, namely, chlorophyll *a*-protein 2 (mol.wt. 51,000), chlorophyll *a*-protein 3 (mol.wt. 43,000), and chlorophyll *a/b*-proteins 1 and 2 (mol.wts. 30,000 and 24,000, respectively). Also present were four polypeptides with molecular weights of 33,000, 22,000, 19,000 and 18,000.

1. INTRODUCTION

The thylakoid membrane in higher plants and algae contains two photosystems each with a specific reaction center plus antennae pigment system (27). Functional identification of the polypeptide components of these two systems

has been attempted using a variety of electrophoretic techniques often in combination with photosynthetic mutants (15, 22, 25, 31, 40). In bacteria, the assignment of polypeptides to the reaction center has been achieved by isolation and direct analysis of the components (16). In

Abbreviations: CF₁ = chloroplast coupling factor; Chl = chlorophyll; Chl-P = chlorophyll-protein; DBMIB = 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCPIP = 2,6-dichlorophenolindophenol; DCMU = 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; F = fluorescence emission; Hepes = *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; HP = high potential; LP = low potential; Mes = 2-(*N*-morpholine)ethane sulfonic acid; Mops = morpholinepropane sulfonic acid; PS = photosystem; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tricine = *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; Tris = tris-(hydroxymethyl)-aminomethane.

higher plant systems such an approach has made possible the assignment of a small number of polypeptides to photosystem I (13, 22, 34). Hitherto, a sufficiently purified oxygen evolving photosystem II particle has not been available to make a similar assignment of polypeptides to this photosystem.

P.-Å. ALBERTSSON, H.-E. ÅKERLUND and B. ANDERSSON (1-7) prepared inside-out vesicles enriched in photosystem II from chloroplast grana. After disrupting and resealing of the grana membranes (28) such inside-out vesicles preferentially partition into a dextran-rich polymer phase. We have optimised the purification of these photosystem II containing and oxygen evolving inside-out vesicles from spinach for a minimum content of thylakoid polypeptides.

2. MATERIALS AND METHODS

2.1. Plant material and chloroplast isolation

Spinacia oleracea L. was obtained from the local market. Intact chloroplasts were isolated in a medium composed of 1.0mM-sodium isoascorbate, 0.35mM-sorbitol, 10mM-MgCl₂ and 50mM-morpholinepropane sulphonic acid (Mops), pH 7.5 (21). The chloroplasts were osmotically lysed by a 10-fold dilution with the above medium containing no sorbitol and the lamellar systems washed by 2 cycles of centrifugation (5 min 12,000 g) and resuspension in the same medium.

2.2. Preparation of PS II vesicles

Mechanical disruption of the photosynthetic lamellae and subsequent fractionation was carried out with an aqueous two-phase (dextran/polyethylene glycol) polymer system according to ALBERTSSON (2) with some modifications. Lamellar systems (1.25 mg chlorophyll·ml⁻¹) in 50mM-Mops (pH 7.5), 1mM-isoascorbate and 10mM-MgCl₂ were disrupted in a French Pressure Cell press (American Instrument Company, Maryland, USA) (flow rate 5ml · min⁻¹) at a pressure of 3,400 lb·in⁻². The pressate was centrifuged at 40,000g for 30 min and the pellet resuspended in 10mM-sodium phosphate (pH 7.4), 5mM-NaCl and 100mM-sucrose to facilitate destacking. Two additional French press treatments of 2,800 lb · in⁻² ensured vesicles of uniform size. The concentration of both poly-

mers during the phase partitioning experiments was 5.95%. The membrane vesicles collected by ultracentrifugation were resuspended in 40mM-NaCl. All operations were carried out at 2.5 °C and all experiments done with freshly prepared vesicles.

2.3. Photochemical assays

Spectrophotometric determinations were carried out with an Aminco DW-2a spectrophotometer operating in either the split-beam or dual-wavelength mode. The samples were irradiated with saturating red light. DCPIP photoreduction was determined as in (21), except that the rate of decrease in absorbance was measured at 550 nm. Photoreduction of methyl viologen was determined by coupling the reaction to the light dependent generation of a proton gradient. This was followed by the use of the pH indicator phenol red with the spectrophotometer operating in the dual-wavelength mode (33).

Oxygen evolution assays were carried out polarographically using a Rank Brothers oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). This was done with 3 different electron acceptors in media composed of (a) 0.35mM-sorbitol, 2mM-EDTA, 5mM-MgCl₂, 50mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (Hepes) pH 7.6, 2.5mM-potassium ferricyanide. (b) 50mM-2-(*N*-morpholine)ethane sulphonic acid (Mes), pH 6.6, 25mM-NaCl, 0.2mM-DCPIP, 10μM-2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) (c) 50mM-sodium phosphate, pH 7.0, 0.5mM-phenyl-*p*-benzoquinone. Photoreduction of methyl viologen was determined by measuring oxygen uptake in a medium composed of 50mM-Tricine, pH 8.4, 35mM-NaCl, 2mM-NaN₃, 10μM-DCMU, 4mM-ascorbate, 0.2mM-DCPIP, 2mM-NH₄Cl, 150μM-methyl viologen.

Simultaneous measurements of proton gradient formation and oxygen evolution were carried out in a Rank Brothers oxygen electrode fitted with a GK 2322C Radiometer Micro combination electrode (Radiometer, Copenhagen, Denmark) in the inner compartment. The combination electrode was inserted through the center plug using air-tight O-ring fittings. The pH changes were monitored using a Radiometer PHM 62 standard pH meter and recorded with a

Servogor 220 Recorder using a full scale sensitivity of 50 mV for the membrane vesicles and 200 mV for the unfractionated thylakoids. The L 409 polarizing adapter and the buffer adjustment screw of the pH-meter was used to adjust the electrical zero. Saturating white light was provided by a Schott KL 150B light source (Schott, Mainz, F.R.G.) equipped with an 8mm fiber optic system. The instrument was calibrated with known amounts of NaOH and HCl.

All photochemical assays were carried out at 20 °C and the chlorophyll concentration was 25 µg chlorophyll·ml⁻¹ unless otherwise specified.

2.4. Absorption and fluorescence emission spectra

Absorption spectra were recorded at room temperature using an Aminco DW-2a spectrophotometer. Fluorescence emissions were recorded at 77K using a fibre optic system (41) with samples placed on filter paper discs.

2.5. Electrophoretic analyses

Samples were prepared for electrophoresis as earlier described (15, 31). Electrophoretic analyses were carried out in a SDS system at 4 °C using a 6 % stacking gel and a 10–15 % linear polyacrylamide gradient separation gel (31). After electrophoretic separation the gels were stained for heme-associated peroxidase activity (24, 44) and/or protein using Coomassie Blue R250.

2.6. Cytochrome determination

Cytochromes were determined from their chemically reduced minus oxidised difference spectra (520–580nm) by the use of an Aminco DW-2a spectrophotometer. From these difference spectra, the cytochromes were quantified based on a millimolar extinction coefficient of 20 cm² · mole⁻¹.

2.6.1. Determination of cytochrome *f* and *b*-559HP

These measurements were carried out essentially as described by BENDALL et al., (12). The

samples were suspended in 50mM-Mes, pH 6.0 at a chlorophyll concentration of 100 µg·ml⁻¹. The redox mediators potassium ferricyanide and hydroquinone were added separately to identical samples to give a final concentration of 1.25 mM. The difference spectrum was recorded after equilibration. The height of the peak at 559nm was taken above a line drawn through the minima at either side of the peak and was corrected for any non-linearity in the baseline. Triton X-100 (1 % final concentration) was added to both cuvettes. This converted the cytochrome *b*-559HP into a form with a lower potential, no longer reducible by hydroquinone (38). The difference spectrum was again measured and now reflects the presence of cytochrome *f* only. Quantification was carried out using the peak height at 554nm. The peak height at 559nm was measured in order to subtract the contribution of cytochrome *f* from the cytochrome *b*-559HP value measured before the Triton addition.

2.6.2. Determination of cytochrome *b*-563 and *b*-559LP

The procedure of RICH and BENDALL was used (38). Each sample was suspended in a medium containing 50mM-Mes, pH 6.0, and 1mM-hydroquinone. Catalase (400 Units·ml⁻¹, Mann Research Laboratories) was included in the reaction mixture to remove H₂O₂ formed during the reaction of dithionite with oxygen. Under these conditions cytochrome *f* and *b*-559HP will be fully reduced. Menadiol freshly prepared as described in (37) was added to the sample cuvette to a final concentration of 1mM. The difference spectrum was recorded and the absorption maximum at 559nm reflects the preferential reduction of cytochrome *b*-559LP. A few crystals of solid dithionite were then added to the measuring cuvette whereas the reference cuvette was made 1mM with respect to menadiol. The difference spectrum was recorded after equilibration and reflects the specific reduction of cytochrome *b*-563.

2.7. Analytical procedures

Chlorophylls were determined in 80 % acetone and quantified according to ARNON (11).

Protein determinations were carried out with the Folin method as modified in (36) with crystalline bovine serum albumin as standard.

2.1 and protein/chlorophyll ratio of 4.3% compared to 3.0 and 7.3, respectively, for the unfractionated membranes.

3. RESULTS

Phase partitioning of the membrane fractions was tested at a series of polymer concentrations ranging from 5.4–6.6%. The chlorophyll content, polypeptide composition and photochemical activities of material partitioning to the polyethylene glycol-rich upper phase and to the dextran-rich lower phase were analysed. Optimal purity of the PS II vesicles was obtained using a two-phase system composed of 5.95% of each polymer.

Table I shows the composition and photochemical activities of the fractions at various stages of the purification procedure. The fraction partitioning to the dextran-rich lower phase was markedly depleted in PS I and enriched in PS II activity corresponding to 9 and 215%, respectively, of the activities seen in the polyethylene glycol-rich upper phase. The total recovery of PS II activity was 38%. The yield of PS II membrane vesicles was approximately 8% on a chlorophyll basis and 4.5% on a protein basis. The PS II vesicles had a chlorophyll *a/b* ratio of

3.1. Spectral analyses

The absorption spectrum of the PS II vesicles at room temperature (Figure 1) reveals an enrichment in chlorophyll *b* while the stroma lamellae (40,000 g supernatant) are virtually free of chlorophyll *b*. The spectrum obtained with material partitioning to the polyethylene glycol-rich upper phase was similar to that obtained with the chloroplast. All the fractions have a chlorophyll *a* red absorption maximum at 680–681 nm.

The low temperature (77K) fluorescence emission spectra of the different fractions obtained during the preparation of the PS II membrane vesicles are shown in Figure 2. Samples of equal chlorophyll concentrations were used to allow direct comparison of the fluorescence yields. It should be noted that the spectrum of the stroma lamellae was recorded after a two-fold expansion as compared with the other samples. The spectrum of chloroplasts showed the characteristic emission peaks at 685, 695 and 730–740 nm. These peaks have

Table I

Characteristics and yield of the fractions obtained during purification of the photosystem II vesicles.

Fraction	Chlorophyll	Protein	Chlorophyll <i>a/b</i>	Protein/ Chlorophyll	Specific Activities	
	(mg)	(mg)			μmoles acceptor	reduced
					Asc/DCPIP→ methyl viologen (PS I activity)	H ₂ O→DCPIP (PS II activity)
Lamellar systems	28.2	205	2.98	7.26	124	59.2
Grana	26	162	2.53	6.25	117	21.4
Stroma lamellae	3.5	22	>7	6.36	228	0.0
Disrupted grana	25.2	137	—	5.40	124	10.7
Vesicles in poly- ethylene glycol phase	5.8	32	3.48	5.47	153	10.7
PS II vesicles in dextran phase	2.2	9.3	2.10	4.29	14	23

Figure 1. Absorption spectra of photosystem II vesicles and of stroma lamellae.

been attributed to emission arising from three principal types of antennae chlorophylls in chloroplasts, namely those of the light-harvesting chlorophyll *a/b*-protein 2, the antennae pigments of PS II and of PS I, respectively (14). The spectral features of the membrane vesicles which partitioned to the polyethylene glycol-rich upper phase are very similar to those of the chloroplasts. In contrast, the emission spectrum of the PS II vesicles had the predominant peak at 695 nm and a $F_{730/695}$ ratio of 0.66 compared to that of 2.8 in the chloroplasts. The emission spectrum of the stroma lamellae shows a single peak at 731 nm.

Thus, fluorescence species distinguishable in the composite spectrum of the thylakoids have been separated during the preparation of the stroma lamellae and the PS II vesicles. This has been accomplished without a blue shift in the F_{695} maximum, as is frequently observed (39) with other methods of fractionation. This suggests that the state of the chlorophylls in the preparation has remained unaltered.

3.2. Photochemical activities

The PS II vesicles exhibited high rates of oxygen evolution with H_2O as the electron donor and using phenyl-*p*-benzoquinone, DCPIP or ferricyanide as terminal electron acceptors (Table II). The measured rates of activities were comparable with those obtained using chloroplasts. This suggests that components involved in these reactions were not depleted and/or inactivated during the isolation procedure. The activities were DCMU sensitive and could be inhibited by Tris washing (0.8 M, pH 8.8). The latter treatment is known to inhibit electron transport on the oxidising side of PS II (18).

The use of the lipophilic electron acceptor phenyl-*p*-benzoquinone ensured that the electron transport was entirely attributable to PS II. This was further substantiated by the use of the plastoquinone analogue DBMIB during the photoreduction of DCPIP. Although ferricyanide is often used as a Hill oxidant it has previously been shown capable of accepting electrons from both photosystems under appropriate conditions (45).

Figure 2. Low temperature fluorescence emission spectra of fractions obtained during preparation of the photosystem II vesicle.

Table II

Oxygen evolution of PS II vesicles compared with other membrane fractions using different electron acceptors ($\mu\text{moles O}_2 \cdot \text{mg}^{-1} \text{ chlorophyll} \cdot \text{hr}^{-1}$).

Assay	PS II Vesicles in dextran phase	Chloroplasts	Vesicles in polyethylene glycol	Stroma lamellae
H ₂ O → phenyl- <i>p</i> -benzoquinone	70– 76	40 – 56	20 – 34.5	0–2
H ₂ O → DCPIP	73– 85	102 –137	48 – 60	0–0
H ₂ O → ferricyanide	160–258	119 –148	137 –171	0–0
H ₂ O → methyl viologen $\mu\text{moles H}^+ \cdot \text{mg chlorophyll}^{-1}$	0–0	0.31– 0.47	0.07– 0.16	0–0

With methyl viologen as the electron acceptor only the chloroplast and membrane vesicles partitioning to the polyethylene glycol-rich upper phase were active. This suggests a more complete electron transport chain in these fractions. In the reactions characteristic of PS II the measured rates of activities seen in the polyethylene glycol-rich upper phase were appreciably lower than the rates seen in the PS II vesicles. As expected, the stroma lamellae were essentially devoid of activity in these determinations.

Figure 3 shows the extent and direction of proton movement in the membrane vesicles obtained after phase-partitioning. The vesicles partitioning to the polyethylene glycol-rich upper phase showed an alkalisation of the medium upon illumination. This photochemical activity is characteristic of broken chloroplasts (35) and suggests that the side orientations of these membrane preparations are comparable. However, the extent of this proton flux was appreciably lower in the membrane vesicles obtained after phase partitioning, amounting to 7.9 nmoles H⁺ · mg⁻¹ chlorophyll as compared with 46 nmoles H⁺ in the chloroplast. Under identical conditions the PS II vesicles showed a net proton extrusion upon illumination corresponding to 6.9 nmoles H⁺ · mg⁻¹ chlorophyll. This indicates

that the PS II vesicles are of opposite sidedness to the original thylakoid membrane (4,5). Because of the time dependent loss in photochemical activities all fractions were measured after completion of the PS II vesicle preparation procedure. The samples were stored at 0–4 °C in the dark during this time.

3.3. Cytochromes

Table III lists the cytochrome composition of the PS II vesicles, the membrane vesicles partitioning to the polyethylene glycol-rich upper phase and the unfractionated membrane. The four cytochromes generally thought to be involved in plant photosynthesis, cytochrome *b*-559HP, *b*-559LP, *b*-563 and cytochrome *f* (17) were present in the fractions albeit in varying amounts. The PS II vesicles were enriched in cytochrome *b*-559HP relative to the other membrane samples and showed an 8-fold increase compared to the membrane vesicles partitioning to the polyethylene glycol-rich upper phase. In contrast, the PS II vesicles were extensively depleted of cytochrome *b*-563 and cytochrome *f*. An enrichment of cytochrome *b*-559LP was found in the polyethylene glycol-phase associated membrane vesicles.

Figure 3. Proton gradient formation and oxygen evolution observed upon illumination of thylakoids (A), vesicles partitioning to the polyethylene glycol phase (B) and photosystem II vesicles (C).

Table III**Cytochrome content in PS II vesicles compared with two other membrane fractions (moles/1000 moles chlorophyll-*a*).**

Fraction	Cytochrome <i>b</i> -559HP	Cytochrome <i>b</i> -559LP	Cytochrome <i>b</i> -563	Cytochrome <i>f</i>
Lamellar systems	1.74	1.27	3.69	1.21
Vesicles in polyethylene glycol phase	0.3	3.22	4.18	1.25
PS II vesicles in dextran phase	2.39	2.36	<0.4	<0.6

Figure 4. SDS-polyacrylamide gel electrophoresis of photosystem II vesicles (a) and vesicles partitioning to the polyethylene glycol phase (b) A: chlorophyll-proteins, B: chlorophyll-proteins as detected by their fluorescence emission (31), C: chlorophyll-proteins and bands stained for heme-associated peroxidase activity using tetramethylbenzidine (44).

The separation was carried out at 4 °C on an 11–15% SDS-polyacrylamide gradient gel. The amount of chlorophyll applied to each slot is indicated (µg). Three steel rods were inserted (track a-30) to mark the positions of the chlorophyll-proteins. The nomenclature system used for the chlorophyll-proteins is defined in (31).

3.4. Electrophoretic analyses

Sodium dodecylsulfate polyacrylamide gel electrophoresis was used to investigate the polypeptide composition of the different membrane fractions obtained in the purification of the PS II vesicles. In the unstained gel the thylakoid preparation routinely showed three principal chlorophyll-containing bands. Identification of these bands was based on a comparison of their electrophoretic mobilities and fluorescence patterns with chlorophyll-proteins identified in barley (31). Like the thylakoids the membrane vesicles partitioning to the polyethylene glycol-rich upper phase showed Chl_a -P1, $\text{Chl}_{a/b}$ -P2 and free pigments in comparable amounts. In contrast, the PS II vesicles contained no (or very little) Chl_a -P1 (Figure 4A). This chlorophyll protein has a low fluorescence yield (Figure 4B) and is thought to be associated with the reaction center of PS I. An enrichment of $\text{Chl}_{a/b}$ -P2 was seen in the PS II vesicles (Figure 4).

Additional chlorophyll-proteins were also observed after the electrophoretic separation, namely: $\text{Chl}_{a/b}$ -P1, $\text{Chl}_{a/b}$ -P2*, Chl_a -P2 and Chl_a -P3 (Figure 4A and 4B). Although the intensities of these bands decreased upon prolonged electrophoresis their relative amounts varied in the different membrane fractions. The PS II vesicles were highly enriched in all these four chlorophyll proteins whereas these bands were markedly depleted or absent from the stroma lamellae. These data are consistent with previous studies implying an involvement of Chl_a -P2 and -P3 in photosystem II (22, 31).

A comparison of the polypeptide composition of membrane vesicles obtained by phase partitioning shows distinct differences (Figures 5 and 6). The PS II vesicles contained eight polypeptides with apparent molecular weights of 51,000, 43,000, 33,000, 30,000, 24,000, 22,000, 19,000 and 18,000. In contrast, the membrane vesicles partitioning to the top phase displayed a more complex polypeptide pattern.

Figure 5. Polypeptide composition of vesicles partitioning to the polyethylene glycol-rich top phase (a) and of the photosystem II vesicles which partitions to the dextran-rich bottom phase (b).

Electrophoretic conditions as indicated in legend to Figure 4. The same amount of chlorophyll (20 μg) is applied to each slot.

Figure 6. Polypeptide composition of the various fractions obtained during the preparation of photosystem II vesicles: a = photosystem II vesicles; b = vesicles partitioning to the polyethylene glycol phase; c = disrupted grana; d = stroma lamellae; e = grana; f = thylakoids.

Electrophoretic conditions as indicated in legend to Figure 4. SSU: small subunit of Ribulose-1,5-bisphosphate carboxylase.

The polypeptide composition of all the various fractions obtained during isolation of the PS II particles are compared in Figure 6. The intensity of the Coomassie Blue stained Chl_a -P1 in the different fractions agrees well with that of the corresponding chlorophyll band (Figure 4A). Chl_a -P2 migrates with an apparent molecular weight of 51,000 (Figure 6). The large subunit of ribulose-1,5-bisphosphate carboxylase and the β -subunit of chloroplast coupling factor (CF_1) have similar mobility. The simultaneous occurrence of the two heavy bands with molecular weights of 51,000 and 12,000 in track d of Figure 6 are thought to represent the large and small subunit of ribulose-1,5-bisphosphate carboxylase. Studies (25) on CF_1 isolated from spinach and barley chloroplasts have shown similar staining intensities of the α - and β -subunits. Thus the contribution of the β -subunit to the staining intensity observed in the 51,000 region can be assessed from the staining intensity of the α -subunit at 59,000. Such a comparison shows that CF_1 is absent (Figure 5) or present in very low amounts (Figure 6) in the PS II vesicles. In contrast, the presence of CF_1 in the remaining fractions is evident from the strong staining of the α -subunit at 59,000. This is also supported by the absence of chlorophyll bands in the 51,000 region in these fractions. The Coomassie Blue bands at 43,000 and 30,000 are located at the positions of Chl_a -P3 and $\text{Chl}_{a/b}$ -P1, respectively, and are enriched in the PS II vesicles compared to stroma lamellae. A polypeptide band corresponding to the apoprotein of $\text{Chl}_{a/b}$ -P2 is seen in all the fractions except stroma lamellae. In addition to the four chlorophyll proteins described above, the PS II vesicles contain four polypeptides of unknown function. These all stain weakly with Coomassie Blue and the two with molecular weights of 33,000 and 18,000 may be the same components as those which are stained with tetramethylbenzidine (Figure 4C).

4. DISCUSSION

A photosystem II vesicle with retained capacity of oxygen evolution has been isolated from spinach chloroplasts using the aqueous two-phase separation of membrane vesicles developed by ALBERTSSON (1, 2). The vesicle showed high

rates of oxygen evolution with PS II electron acceptors, little PS I activity, an enrichment in cytochrome *b*-559HP, a depletion of cytochrome *b*-563, reversed proton pumping, a low F735/F695 ratio and a simple polypeptide composition. Addition of DCMU (Figure 3) or Tris washing (0.8M, pH 8.8) abolished oxygen evolution and proton gradient formation.

PS II vesicles of varying purity have been isolated from algae, cyanobacteria and higher plants but these have all been inactive with respect to H_2O -splitting activity (10, 30, 39). Their photochemical characterization was therefore based on measurements using the artificial electron donor diphenylcarbazide. Recently, a PS II particle capable of H_2O -splitting has been isolated from the thermophilic cyanobacterium *Phormidium laminosum* (43). In contrast to the spinach vesicle it was more complex with respect to cytochrome content and polypeptide composition.

The cytochrome content of the PS II vesicle herein reported is in agreement with earlier fractionation studies indicating a non-uniform distribution of the cytochromes. Although the exact localization of cytochrome *b*-563 (E_m -110mV) and cytochrome *b*-559LP (E_m +20mV) is unknown, they are thought to function in cyclic electron flow around PS I and II, respectively (17, 20). Cytochrome *f* (E_m +375mV) is associated with PS I (17) and cytochrome *b*-559HP (E_m +380mV) is thought to be closely involved on the oxidising side of PS II (23). Ageing of the chloroplasts is accompanied by the conversion of cytochrome *b*-559HP to a form of lower potential (23, 38) and could have resulted in an over-estimation of cytochrome *b*-559LP present in the PS II particle.

The reversed proton pumping of vesicles partitioning to the dextran-rich lower phase upon phase-partitioning was earlier demonstrated by ANDERSSON, ÅKERLUND and ALBERTSSON (4). The PS II vesicles in the present study obtained have the same characteristics and thus appear to have been derived from grana stacks in accordance with the proposed mechanisms (5, 7).

The low temperature fluorescence emission spectrum of the PS II vesicles contained a principal peak at 695nm. One molecular species which emits at this particular wavelength is thought to belong to the antenna of the PS II

reaction center. In accordance, an increased emission at this wavelength could be expected in a PS II vesicle. A blue shift of this component was observed in PS II particles studied earlier and has been attributed to an alteration in the molecular environment of the F695 component. The emission spectrum of the stroma lamellae was confined to one peak at 731 nm. It has thus been possible to separate the short and long wavelength components of the composite emission spectrum of the chloroplasts without alteration of the fluorescence patterns.

The properties of the PS II vesicle described above are identical with those properties of freshly isolated chloroplasts which have been attributed to PS II. Retention of these characteristics appears to be related to the omission of detergents during its preparation. For instance, loss of the H₂O-splitting activity (47), alteration in certain spectral characteristics (26) and an alteration of the redox potential of cytochrome *b*-559HP (38) are seen upon addition of the comparatively mild non-ionic detergent Triton X-100 to isolated chloroplasts. Furthermore, a direct correlation between amount of PS II H₂O-splitting activity and the E_m of cytochrome *b*-559HP has been demonstrated (23). This correlation is supported by the present data.

Sodium dodecylsulfate polyacrylamide gel electrophoresis under relatively mild conditions (31) revealed the presence of four chlorophyll-proteins in the PS II particle. These have been identified as Chl_a-P2, Chl_a-P3, Chl_{a/b}-P1 and Chl_{a/b}-P2. All these chlorophyll-proteins have earlier been assigned to PS II by the use of photosynthetic mutants and by fractionation studies (31). The identity of the four remaining polypeptides is unknown although two of these appear to stain with tetramethylbenzidine. The weak staining intensity of these polypeptides with Coomassie Blue could indicate a weak staining affinity or their presence in less than stoichiometric amounts compared to the chlorophyll-proteins. One of these polypeptides has an apparent molecular weight in the region of 32,000. Since the photosystem II vesicles are sensitive to DCMU, this polypeptide could be the shielding protein which binds DCMU (32). A manganese-containing protein involved in oxygen evolution has been isolated from spinach chloroplasts (42). This protein has a molecular

weight of 65,000 and should be contained in the PS II particle but could not yet be set in relation with certainty to any of the observed polypeptide bands. Photochemical measurements also indicate the presence of high amounts of cytochrome *b*-559HP in the vesicle. This cytochrome has been reported to be composed of subunit polypeptides with molecular weights around 6,000 (49). It has also not been possible to assign a specific polypeptide band of the PS II particle to this cytochrome. Photosystem II membranes isolated from a mutant of *Chlamydomonas* (19) deficient in coupling factor and PS I show the presence of Chl_a-P2 and Chl_a-P3 in addition to several other unidentified components. Since they show similar electrophoretic mobilities, these two chlorophyll-proteins may also be regarded as identical with components of a PS II particle recently isolated from the cyanobacterium *Anacystis nidulans* (30).

Although the procedure of phase-partitioning was previously used to isolate inside-out membrane vesicles from chloroplast grana (4–6), electrophoretic analyses showed that such vesicles contained polypeptides attributable to both photosystems. In these earlier analyses, the procedure was directed towards the isolation of vesicles of opposite sidedness whereas the goal of the present study was to obtain a PS II vesicle with no PS I contamination. One possibility is that these PS II vesicles are derived more strictly from the appressed regions of the granal stacks. This would be in agreement with the suggestion of the exclusive location of PS II components together with the light-harvesting chlorophyll *a/b*-protein in the appressed regions (8, 9). Alternatively, the detergent-like properties of polyethylene glycol (29, 46) in the higher concentrations used may have resulted in the extraction of PS I components from the membrane vesicles in the bottom phase and their partitioning into the upper phase.

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