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The minor antenna complexes of an oxygen evolving photosystem II preparation: purification and stoichiometry

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A photosystem II complex obtained by partial solubilization of spinach PS II membranes has been analyzed in terms of chlorophyll-protein complexes. The complex was found to contain the minor antennas CP29 and CP26 and a very small amount of LHC II (less than 3%). No trace of CP24 was found to be associated with the PS II complex. This chlorophyll *a*/*b*-protein was instead found in the fraction containing the main light-harvesting complex LHC II. The stoichiometry of the minor antennas indicates the values 3:3:1 for the ratios CP29:CP26:P680.

Photosystem II; Chlorophyll-protein; Minor antenna; Stoichiometry; (Spinach)

1. INTRODUCTION

During the last few years a number of significant advances have been achieved in the knowledge of the structure-function relationship of photosystem II. A major advance was the isolation by Nanba and Satoh [1] of a PS II particle with charge separation activity containing only the D1 and D2 apoproteins plus cytochrome *b*-559. The presence of a light-induced spectral change due to pheophytin reduction and the appearance of a spin-polarized triplet at cryogenic temperature leave no doubt on the P680 being actually contained in this particle [2]. More recently Seibert et al. [3,4] were able to measure the kinetics for charge separation in the PS II particles by picosecond spectroscopy and Barber et al. [5] succeeded in restoring the presence of the primary stable acceptor Q_A in this preparation. These results definitely clarify the identity of the PS II reaction center showing that the D1 and D2 apoproteins hold P680, pheophytin and the quinone acceptors in the very same way as the bacterial reaction center polypeptides L and M [6,7]. On the other hand, the notion has clearly emerged that the PS II organization in higher plants is far more complex than in bacteria and is also more complex than that of PS I. Thus, besides the reaction center particle (D1, D2 and cytochrome *b*-559) on one side and the main light-harvesting system (LHC II) on the other, at least five chlorophyll-protein complexes have been identified whose functional role is still unclear. These are the two complexes CP47 and CP43 which contain only chlorophyll *a* and the three complexes CP29 [8,9], CP26 [10,11] and CP24 [12] which also contain chlorophyll *b*.

Ghanotakis et al. [13,14] obtained a PS II preparation through solubilization of PS II membranes by octylglucoside (*n*-octyl *β*-D-glucopyranoside), which was able to evolve oxygen and was partially sensible to DCMU inhibition. This preparation was a more integrated system with respect to the simple reaction center particle ob-

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Ioxynil, 3,5-diiodo-4-hydroxybenzonitrile; OEE, oxygen evolving enhancer; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; Tricine, *N*-(tris[hydroxymethyl]-methyl)-glycine

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tained by Satoh et al., and yet was free from the large excess of pigments carried by LHC II. It therefore appears a particularly suitable system to investigate the structure-function relationship between the reaction center (D1, D2, cytochrome *b*-559) and the other chlorophyll-protein complexes.

This paper reports a revision of the analysis of the PS II complex as prepared by Ghanotakis et al. [13], and provides the stoichiometric relationships between the P680 reaction center and the minor chlorophyll *a/b*-protein complexes. This preliminary characterization is essential to the investigation of the functional role of the chlorophyll *a* and chlorophyll *a/b* internal antennas of PS II.

2. MATERIALS AND METHODS

2.1. *Spinach thylakoids, PS II membranes, and PS II complex preparation*

Spinach thylakoids and PS II membranes were isolated as described [15,16]. PS II complex was prepared as in [13,14], using octylglucoside to solubilize PS II membranes in the presence of high ionic strength.

2.2. *Sucrose gradient ultracentrifugation*

PS II complex was resuspended at 0.8 mg chl/ml in 5 mM Hepes, pH 7.2, 1 mM EDTA and then solubilized for 5 min at 0°C in the presence of 0.4% (w/v) dodecylmaltoside (*n*-dodecyl β-D-maltoside). Unsolubilized material was removed by centrifugation at 15000 × *g* for 10 min. The supernatant (6 ml) was loaded onto a 32 ml 0.1–1.0 M sucrose gradient containing 5 mM Hepes, pH 7.2, 0.06% DM and run at 28000 rpm in a Kontron TST 28.38 rotor at 4°C for 18 h. For analytical purposes 0.3 ml aliquots were loaded onto 4 ml 0.1–1.0 M sucrose gradient and run at 56000 rpm in a Kontron TST 60.4 rotor at 4°C for 6 h.

2.3. *Non-denaturing isoelectrofocusing*

The upper band obtained from the sucrose gradient was collected and concentrated by dialysis against solid sucrose to a chlorophyll concentration of 1 mg/ml. A 1 mg aliquot was subjected to non-denaturing isoelectrofocusing analysis, on an 18 ml bed of Ultrodex (LKB) prepared as described [17]. The sample was focused at a constant power of 3 W for 14 h at 4°C. The green bands resolved were then eluted from small plastic columns with a small volume of 25 mM Hepes, pH 7.2, 0.06% dodecylmaltoside.

2.4. *Gel electrophoresis*

Samples were solubilized in 5% SDS, 2.5% β-mercaptoethanol, 4 M urea, 62.5 mM Tris-H_2SO_4, pH 9.0. Aliquots of 2 μg of chlorophyll for isolated chlorophyll-protein complexes and of 8 μg for thylakoids, PS II membranes and PS II complex were loaded onto a 12–18% acrylamide gradient gel containing 6 M urea [10].

2.5. *Immunological assays*

The proteins resolved by denaturing electrophoresis were blotted onto a nitrocellulose filter [18] and then assayed with polyclonal antibodies raised against pure preparations of LHC II and PS I 200 [19] from maize.

The immunoreactions were detected using a secondary antibody conjugated with horse peroxidase (Sigma), whose enzymatic activity was developed by using H_2O_2/3-amino-9-ethylcarbazole as substrate [20].

2.6. *Herbicide binding*

To determine the antenna size of PS II complex 125 μl of a solution of the complex (0.72 mg/ml in terms of chlorophyll) were added to 1 ml aliquots containing different concentrations of [^{14}C]ioxynil (9.5 Ci/mol) in 50 mM tricine, pH 7.3, 15 mM NaCl, 5 mM $MgCl_2$ and incubated in the dark for 5 min at 20°C. The samples were then centrifuged in a microfuge at 12000 × *g*. Aliquots of the supernatants were withdrawn and counted in a liquid scintillation counter (Beckman LS1800). The amount of herbicide bound to the reaction centers was calculated by difference with control samples obtained by exactly the same procedure except for the presence of the PS II complex.

2.7. *Other methods*

Chlorophyll concentration and *a/b* ratio were determined as in [21]. Protein concentration was determined with the bicinchoninic acid protein assay (Pierce) [22], using bovine serum albumin as standard.

3. RESULTS

When subchloroplast PS II membranes are treated with octylglucoside under the experimental conditions defined by Ghanotakis et al. [13,14], a PS II particle named 'PS II complex' is obtained which retains a high oxygen evolving activity. This complex is collected as the supernatant of a centrifugation step whose pellet contains the bulk LHC II fraction and some of the PS II complexes which were not extracted in the solubilization step. This pellet will be referred to as 'fraction L'. The supernatant is then subjected to a dialysis step to eliminate detergent, sucrose and sodium chloride. A second centrifugation step allows collection of concentrated PS II complex as the pellet.

Fig.1 shows the polypeptide composition of the PS II complex compared to that of the fraction L and of the PS II membranes. In the PS II complex a number of polypeptides can be easily recognized as the apoproteins of CP47 and CP43, the hydrophilic 33 kDa protein OEE1, the two polypeptides D1 and D2 and the two subunits of cytochrome *b*-559. Besides these apoproteins at least three polypeptides which are of more difficult

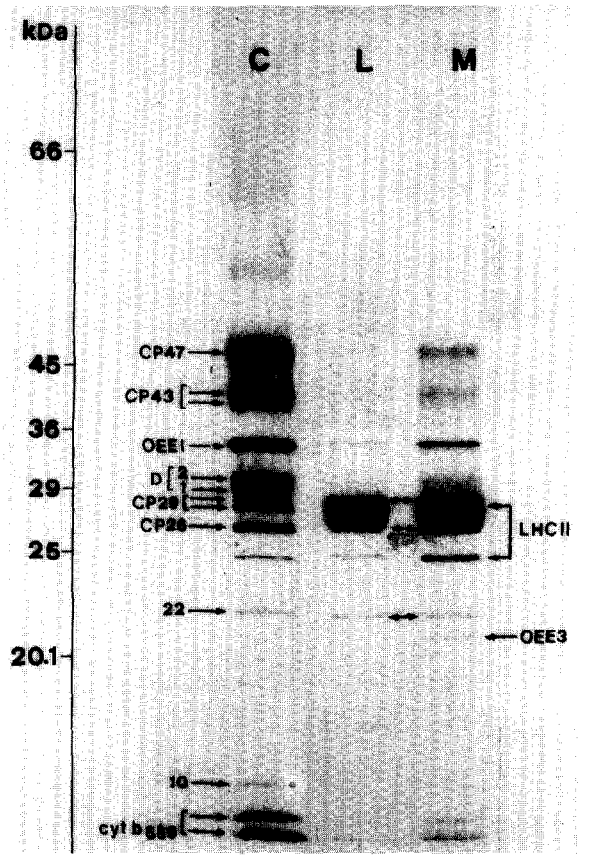

Fig.1. Denaturing SDS-PAGE showing the polypeptide composition of PS II complex (C), fraction L (L) and PS II membranes (M). The band at 22 kDa present in line C does not represent the CP24 apoprotein (see also fig.2) but the structural polypeptide identified by Ljungberg et al. [22]. At approximately the same position the CP24 apoprotein is instead visible in lines L and M (marked with double arrow). The two other double arrows identify the position of CP26 and CP29 apoproteins.

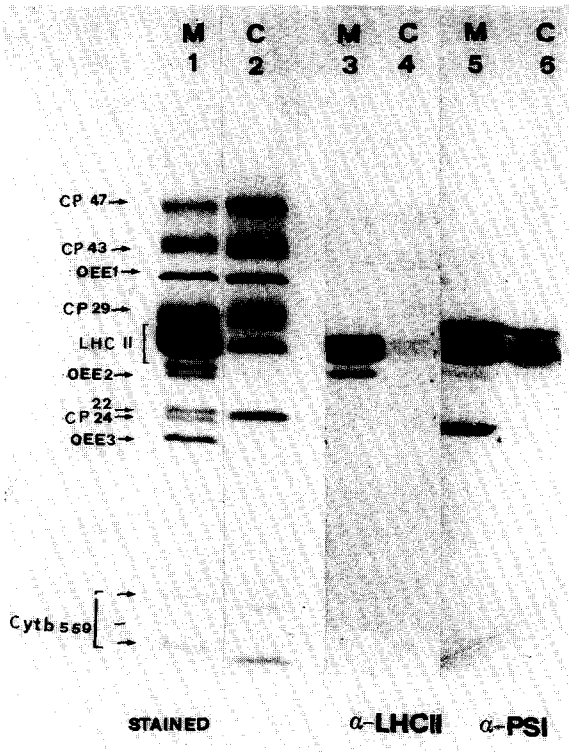

Fig.2. Western blot analysis of PS II membranes (M) and PS II complex (C). Lines 1 and 2 represent the denaturing SDS-PAGE and lines 3–6 the immunoblots using two polyclonal antibodies (see text). α-LHC II recognizes the LHC II polypeptides in the PS II membranes (line 3) and a faint trace of the same polypeptides in the PS II complex (line 4). α-PS I recognizes CP29, the LHC II polypeptides, CP26 (superimposed to the LHC II apoproteins) and CP24 in the PS II membranes (line 5). In the PS II complex only CP29 and CP26 (and traces of LHC II apoproteins) are recognized (line 6).

attribution are clearly visible in the range of apparent molecular mass 26–30 kDa. In fact they could be apoproteins of the minor antennas CP29 and CP26 or polypeptides belonging to LHC II [10]. The 23 kDa polypeptide OEE2 is also found to an extent which varies in different experiments. The latter phenomenon is possibly due to partial recombination of this component to the PS II complex during the low ionic strength conditions which are realized in the dialysis step. Two polypeptides whose functional role is still uncertain, are observed at 22 and 10 kDa [23]. The presence of CP43 as a doublet was formerly observed [14] and attributed to either a phosphorylated or a partially digested form of the same apoprotein.

A 'Western blot' analysis using two polyclonal antibodies against purified preparation of LHC II (α-LHC II) and PS I 200 (α-PS I), respectively, was performed to resolve the attribution of the polypeptides in the 26–30 kDa range. α-LHC II is strongly specific for its antigen. Very low cross-reactions with other apoproteins of chlorophyll *a/b* protein complexes, although observed in other experiments, could not be revealed under the conditions used in this analysis. α-PS I cross-reacts

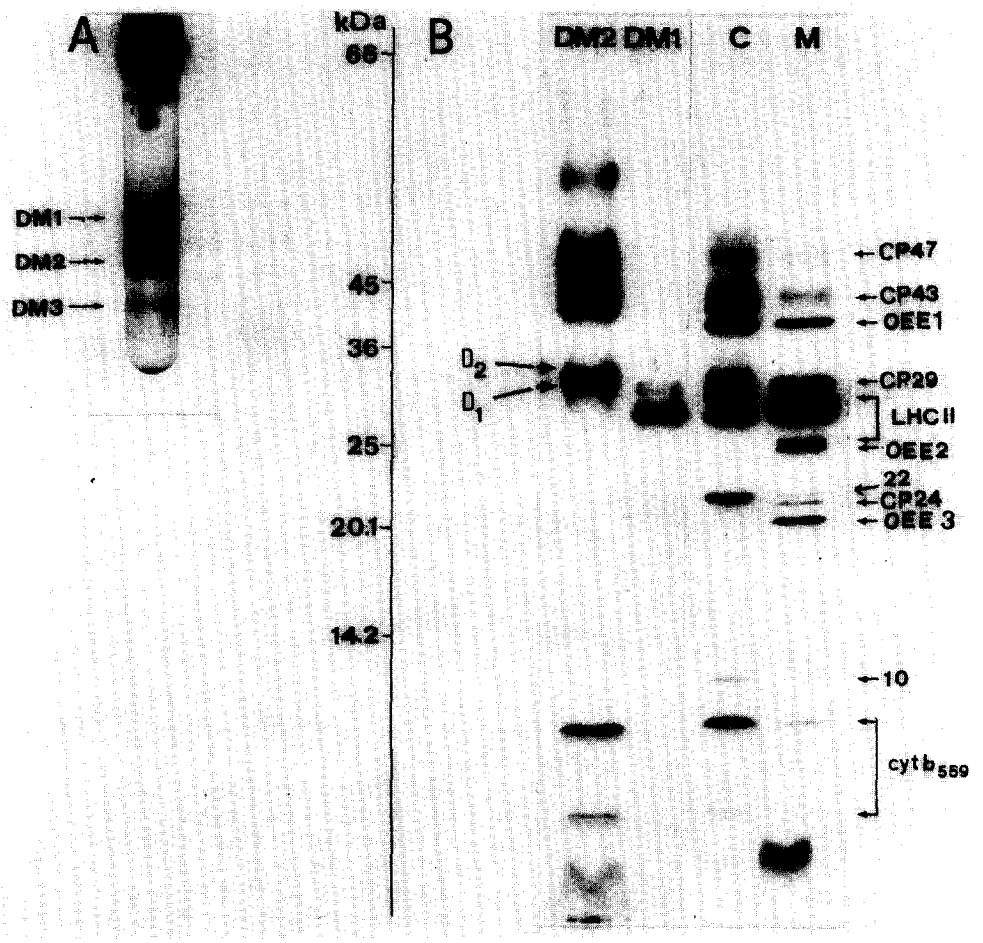

Fig.3. (A) Sucrose density gradient fractionation of PS II complex. (B) Polypeptide composition of the DM1 and DM2 fractions compared with the PS II complex (line C) and PS II membranes (line M). The polypeptide composition of the DM3 fraction (not shown) was identical to that of DM2.

with both LHC II and the minor chlorophyll *a/b* antennas CP29, CP26 and CP24.

The results of the immunoblots are shown in fig.2. In line 4 a very faint reaction of α-LHC II with the PS II complex is shown documenting the presence of extremely low amounts of LHC II polypeptides not even detectable by Coomassie staining (line 2). On the other hand α-PS I clearly recognizes at least two polypeptides (possibly three) which cannot belong to LHC II and must therefore be attributed to CP29 and CP26. No trace of the third minor antenna CP24 is found in PS II complex (line 6) which instead is clearly demonstrated in the PS II membranes (line 5).

The PS II complex was further fractionated by ultracentrifugation in sucrose density gradient after solubilization in dodecylmaltoside. Three green bands were obtained whose polypeptide composition is shown in fig.3. The first green band (DM1) was found to contain the minor antennas CP29 and CP26 (associated with the little amount of LHC II still present in the PS II complex). The second green band (DM2) contains all the remaining polypeptides belonging to the reaction center and its chlorophyll *a* internal antennas (CP47 and CP43). The third green band (DM3), present in a much lower amount, shows the same polypeptide composition as DM2 (not shown) and must therefore be regarded as an aggregated state of DM2. This result is confirmed by the immunoblot

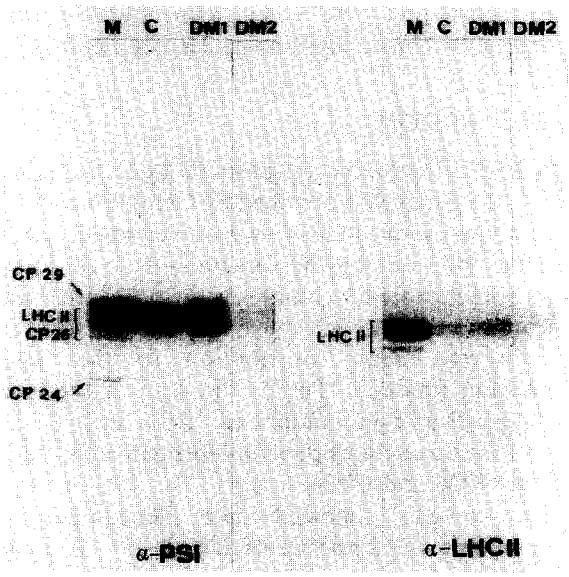

Fig.4. Immunoblot analysis with two polyclonal antibodies of the SDS-PAGE reported in fig.3. Symbols as in fig.3.

analysis in fig.4. In particular it is worth noting that the residual LHC II bound to PS II complex is only found in DM1 fraction associated with the minor antenna complexes. The analysis of the total chlorophyll associated with each sucrose density green band shows that 28% of the total chlorophyll is associated with DM1, the remaining 72% being associated with DM2 and DM3.

The green band DM1 containing the minor antennas of the PS II complex was subjected to isoelectrofocusing in the presence of dodecylmaltoside following the procedure in [17]. Seven fractions were obtained in the pH range 3.5–5.0 whose polypeptide composition is given in fig.5. The fractions 3 on one side, and 6 and 7 on the other, are found to contain CP26 and CP29, respectively, in a very pure form. Fractions 4 and 5 contain both complexes in different proportions, fractions 1 and 2 contain the small amount of LHC II which was still found to be associated with the PS II complex.

Taking advantage of the possibility of having

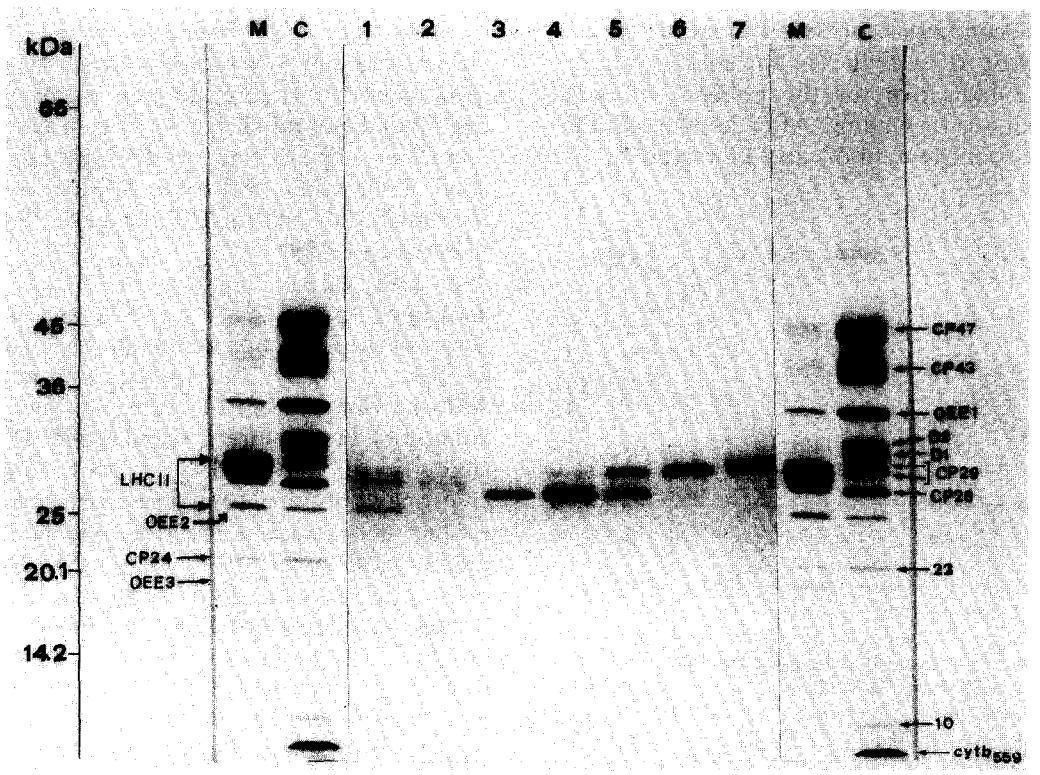

Fig.5. Polypeptide composition of the seven isoelectrofocusing fractions in which the PS II complex was resolved (see text). The polypeptide composition of the PS II membranes (M) and PS II complex (C) are reported for comparison.

the minor antenna complexes in a pure form, in order to state the stoichiometric relationships between the components of the PS II complex, we have measured the chlorophyll *a*/chlorophyll *b* and chlorophyll/protein ratios for the various chlorophyll-protein complexes described above. The results are given in table 1. From these data the stoichiometric ratio between the two minor antennas in the DM1 sucrose density fraction can be calculated as well as the stoichiometric ratios between minor antennas and the reaction center (P680) (see below).

To support these results we have measured the antenna size of our PS II complex by an independent method. This consisted of measuring the number of reaction centers through the binding of a radioactive labelled herbicide. We used ioxynil herbicide which is known to prevent electron transport from Q_A to Q_B and to bind reaction center II in a 1:1 stoichiometric ratio. We preferred this herbicide rather than atrazine or diuron since its affinity is less decreased after detergent extraction [24]. The results are reported in fig.6. Under the conditions used the saturation curve is characteristic of a tight binding with a sloping asymptote indicative for the presence of some (~15%) unspecific binding. After suitable subtraction of the contribution of unspecific binding, the equivalence point for a 1:1 stoichiometry was found at 88 chlorophyll molecules per P680.

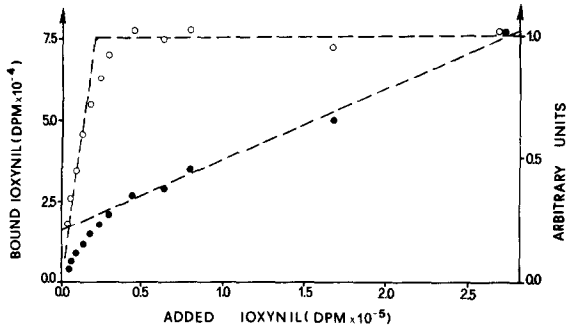

Fig.6. Saturation curve for the binding of the herbicide ioxynil to the PS II complex (closed symbols). The open symbols represent the data after normalization to the sloping asymptote. The asymptote was obtained as least-squares linear regression on the points whose abscissa is higher than 5×10^4 dpm. The equivalence point on the normalized curve is at 2.15×10^4 dpm corresponding to 11.3 nmol of [^{14}C]ioxynil per μmol of chlorophyll. Under the experimental conditions used (see section 2) this corresponds to 88 molecules of chlorophyll per reaction center assuming a stoichiometry of 1:1 for the herbicide binding.

4. DISCUSSION

A PS II reaction center preparation depleted of LHC II, such as that obtained by Ghanotakis et al. [13], and reproduced here, was proved particularly suitable for studying the functional role of those chlorophyll-protein complexes which are not essential to the photochemical activity and yet give a relatively small contribution to the light harvesting function. This is the case of the two chlorophyll-protein complexes CP47 and CP43 and of the three chlorophyll *a*/*b*-protein complexes CP29, CP26 and CP24.

The results reported here unequivocally demonstrate the presence of CP29 and CP26 in association with the PS II core complex constituted by the six apoproteins cytochrome *b*-559 (9 and 4 kDa), D1, D2, CP47 and CP43. Similar results were reported by Ghanotakis et al. [14] who resolved the dodecylmaltoside solubilized PS II complex into two fractions by gel filtration chromatography. However, while their major component 'PS II core complex' (peak A) exactly corresponds to our sucrose density fractions DM2 and DM3, a disagreement exists regarding their second minor component (peak B) which is reported to contain only chlorophyll *a* and is tentatively attributed to CP29.

It also clearly appears that CP24 is not associated to the PS II complex and is instead found in the L fraction containing the main antenna LHC II (fig.2). This finding is in accordance with the view that CP24 could play the role of a connection between LHC II and the PS II complex similarly to LHC I-680 in PS I. These two chlorophyll-protein complexes are indeed very similar both from a spectroscopic and an immunological point of view [10,19]. From the chlorophyll/protein and chlorophyll *a*/chlorophyll *b* ratios measured for CP26 and CP29 (table 1) the number of chlorophyll *a* and chlorophyll *b* associated with each of the two pure complexes was calculated. The resulting values are also reported in table 1. Using these data and the measured chlorophyll *a*/chlorophyll *b* ratio of the DM1 sucrose gradient fraction, the ratio of the two complexes in the DM1 fraction can be calculated

Table 1

Chlorophyll *a*/chlorophyll *b* and chlorophyll/protein molar ratios of different chlorophyll-protein complexes of photosystem II

	Chl/P	Chl*a*/Chl*b*	Chl*a*[a]	Chl*b*[a]
CP26	5.04 ± 0.69	2.71 ± 0.21	3.65	1.35
CP29	4.02 ± 0.77	4.34 ± 0.55	3.27	0.75
DM1	3.90 ± 1.00	3.19 ± 0.15		
DM2	57 ± 5			

[a] Values calculated

The Chl/P values were calculated assuming a molecular mass of 30 kDa for CP26 and CP29 and 160 kDa for the PS II core complex (DM2). The reported values correspond to the average of five measurements

giving the value of 1.3. Taking into account the experimental errors this value is compatible with a stoichiometric ratio of 1:1 for the two minor complexes CP29 and CP26 in the DM1 green band of the sucrose density gradient.

Using the same data the chlorophyll/protein ratio for the entire DM1 fraction can be calculated giving the value 4.5. This compares favorably with the measured value of 3.9 ± 1 showing internal consistency of the data reported in table 1.

Assuming a molecular mass of 160 kDa for the PS II core complex contained in the DM2 fraction (i.e. the complex formed by cytochrome *b*-559, D1, D2, CP47 and CP43) as quoted by various authors [25,26], a value of 57 chlorophyll *a* molecules per reaction center is obtained in agreement with the antenna size measured by Yamada et al. [27].

Finally, taking into account that the chlorophyll associated with the PS II core complex (DM2 + DM3 in the sucrose density gradient) is 72% of the total chlorophyll of the PS II complex we may calculate that 22 chlorophyll molecules are associated to the ensemble formed by the two minor antennas CP26 and CP29 (DM1 fraction). We neglect the contribution of the residual LHC II associated with the PS II complex (lines 1 and 2 in fig.5) which, however, does not represent more than 3% of the total chlorophyll.

Since the couple CP29 + CP26 contains 9 chlorophyll molecules (see table 1) we end up with a stoichiometric ratio of 2.5 CP29 + CP26 complexes per reaction center. If we interpret this figure as 2 CP29 plus 2 CP26 per reaction center the total antenna size for the PS II complex would result 75. A value of 3 would give an antenna size of 84. The herbicide binding experiment reported in fig.6 yielded an antenna size of 88 in very good agreement with the data reported above in the hypothesis of 3 minor antennas per reaction center. This finding also agrees with the well established trimeric structure of the main antenna LHC II [28–30], suggesting a possible trimeric structure for CP29 and CP26 as well.

The third minor antenna CP24 is not found in association with the PS II complex but rather with the main LHC II fraction. On the basis of its striking similarity with the PS I antenna LHC I-680, which has been proposed to act as a connecting unit between the PS I reaction center and the phosphorylatable 'mobile' population of LHC II [19], it could be speculated that CP24 plays a similar role in PS II, connecting the same phosphorylatable mobile population to the PS II complex.

A different LHC II population (up to 5–6 different populations have been separated by isoelectrofocusing [31]) could be strongly associated to CP43 at the site where the 'non mobile' bulk LHC II is connected to the PS II core complex. This would account for the small amount of LHC II found in our PS II complex preparation.

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