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Chlorophyll levels in the pigment-binding proteins of photosystem II

A study based on the chlorophyll to cytochrome ratio in different photosystem II preparations

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The chlorophyll levels in pigment proteins of photosystem II were investigated by using photosystem II preparations with different levels of complexity. Based on the assumption that there is 1 cytochrome *b*559 per reaction centre it has been found that oxygen-evolving complexes containing CP26 and CP29 bind 42 chlorophyll molecules. When CP26 and CP29 are stripped away, the resulting PSII cores bind 30 chlorophyll molecules while CP43-less cores bind approximately 18 chlorophylls. It is therefore concluded that CP47 and CP43 bind 9–12 molecules of chlorophyll *a* and the D1/D2 complex binds 6 chlorophylls. Taken together CP26 and CP29 bind about 12 chlorophyll molecules.

Photosystem II; Chlorophyll-protein; CP47; CP43; Pigment level; Spinach

1. INTRODUCTION

Photosystem (PS) II is a large multi-subunit protein complex, universally present in thylakoids of oxygenic organisms. Both in cyanobacteria and in chl*b*-containing organisms, PS II seems to be organized according to the same basic scheme. The primary electron donor, P680, together with other immediate electron donors and acceptors, is bound into the so-called D1/D2/cyt *b*559 complex [1,2]. This reaction centre complex is associated with other polypeptides, two of which are chl*a*-binding polypeptides and called CP47 and CP43 because of their apparent mobility after non-denaturing SDS-PAGE [3]. Particles with this polypeptide composition, usually defined as PS II core complexes, have been isolated from thylakoids of a variety of organisms [4–8] using different methods. These findings have led to the concept that the PS II core complex is present in the thylakoid membrane as a physically stable and chemically defined lipoprotein unit, identified in higher plants and green algae as the EFs particles observed after freeze fracture of the thylakoid membrane [9]. Moreover, the association of the 33-kDa extrinsic protein with the PS II core complex is believed to form the smallest stable unit still able to perform oxygen evolution [10,11].

The isolation of PS II reaction centres (RC) containing the D1, D2 and cyt *b*559 polypeptides, ruled out a direct involvement of both CP47 and CP43 in PS II photochemistry, assigning them to an antenna function only. At present, however, other functions for these complexes, besides light harvesting, cannot be excluded, although only very indirect evidence exists about a role for CP43 in stabilizing quinone binding [12].

Although the existence of CP47 and CP43 have been recognised for some years [13] and their primary structures known from DNA sequencing [14,15], some basic information is still lacking. For example, it is not known how many pigments are bound to each polypeptide. This is due to large discrepancies observed when the antenna size of the core complex is measured by different methods, such as Q_A photoreduction, pheophytin or cyt *b*559 content.

In this paper we report on the possible number of pigments associated with CP47 and CP43 as deduced from chlorophyll/cyt *b*559 ratio in PS II preparations obtained by selective solubilization of CP47 and CP43 isolated from PS II cores without loss of cyt *b*559. Our data suggest that 9–12 chl molecules could be bound to each CP43 and CP47 polypeptide.

Abbreviations: PS, Photosystem; cyt, cytochrome; SDS-PAGE, sodium dodecylsulphate gel electrophoresis; RC, reaction centre; HPLC, high-pressure liquid chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; DM, *n*-dodecyl-*β*-D-maltoside; OG, *n*-octyl-*β*-D-glucopyranoside; OTG, *n*-octyl-*β*-D-thioglucopyranoside; chl, chlorophyll; pheo, pheophytin

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2. MATERIALS AND METHODS

2.1. *Isolation of PS II-enriched membranes and oxygen-evolving PS II complexes*

PS II-enriched membranes of spinach thylakoids were obtained ac-

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cording to [16] with modifications described in [17]. A highly oxygen evolving PS II preparation was obtained by solubilization of PS II-enriched membranes with the non-ionic detergent *n*-octyl-β-D-glucopyranoside (OG) in the presence of high ionic strength as described in [11].

2.2. *Isolation of PS II subcomplexes*

To obtain a PS II core complex devoid of any chl*a*/*b*-binding proteins, the oxygen-evolving PS II complex described above was resuspended in 10 mM HEPES (pH 7.5), containing *n*-dodecyl-β-D-maltoside (DM) at a final concentration of 0.5%. One milliliter of the suspension of the solubilized PS II complex corresponding to 0.5 mg chl, was loaded onto a 0.1–1.0 M linear sucrose density gradient containing 10 mM HEPES pH 7.5 and 0.03% DM. Gradients were spun for 18 h at 39000 rpm at 4°C in a Beckman SW41 rotor. To isolate a CP43-depleted core, we used a modification of the Akabori et al. method [18]. The PS II complexes obtained by the method of Ghanotakis et al. [11] were resuspended in 10 mM HEPES pH 7.5 to which 20 mM *n*-octyl-β-D-thioglucopyranoside (OTG) was added. After 1 h solubilization with occasional stirring, 1 ml aliquots were loaded onto a 0.1–1.0 M sucrose linear gradient containing 20 mM OTG and 10 mM HEPES pH 7.5. Gradients were spun at 39000 rpm for 24 h at 6°C using a Beckman SW 41 or Kontron TST 41 rotor. Another CP43-depleted PS II preparation was isolated using the anionic exchange chromatography method described in [19]. D1/D2/cyt *b*559 PS II RC complexes were isolated as described both in [2] and [19], except in the former case Triton X-100 was exchanged with DM during the second chromatographic step [20].

2.3. *Isolation of CP47 and CP43*

CP47 was isolated as described in [19]. Highly enriched CP43 fractions were obtained as a byproduct during PS II RC isolation following the procedure in [19]. During the loading of the first anionic column, CP43 does not bind and is eluted. Some of the fractions were collected and CP43 purified by a second anionic exchange chromatography step, changing the pH of the buffer to 7.2. Under these conditions CP43 binds to the column and is eluted with 30 mM $MgSO_4$ without contamination from other polypeptides.

2.4. *Other methods*

HPLC analyses were performed as described in [20]. SDS-PAGE in the presence of 6 M urea was carried out according to [21]. Determination of Coomassie binding following the procedure in [22]. Chlorophyll concentration was determined according to [23]. Cytochrome *b*559 content was calculated from its chemically induced (ferricyanide-oxidised minus dithionite-reduced) difference spectrum, using 17.5 $mM^{-1} \cdot cm^{-1}$ as extinction coefficient [24].

3. RESULTS

3.1. *Characterisation of the oxygen-evolving PS II complex*

Polypeptide composition of the oxygen-evolving PS II complex obtained according to [11] is shown in Fig. 1, lane 1. Identification of polypeptides found in this preparation has been previously reported [25,26]. Table I shows results from a typical HPLC analysis of the pigment levels of the PS II complex. Assuming 2 pheo per P680, 42 chl*a*, 8 β-carotene and 1.5 plastoquinone molecules, have been determined. Moreover, when the chl/cyt ratio was calculated from absorption measurements, 42 ± 4 chls were found (Table II), indicating the presence of 1 cyt *b*559 per 2 pheo, i.e. 1 cyt *b*559 per P680 as previously reported in [27].

To test whether the detergents we employed for subsequent PS II fractionation had any side effect on

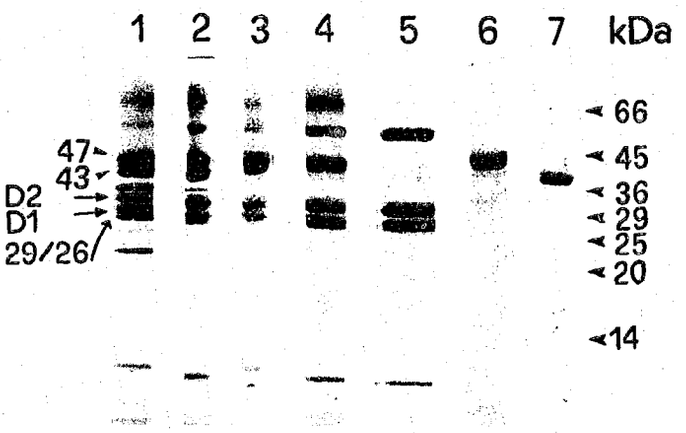


Fig. 1. Polypeptide composition of various PS II preparations and isolated PS II subunits. Lane 1, oxygen evolving PS II complex; lane 2, PS II core complex (DM1 band); lane 3, CP43-depleted PS II core complex (OTG2 band); lanes 4, 5 and 6, CP43-depleted PS II core, PS II reaction centres and CP47 obtained as described in [19]; lane 7, CP43.

the relative levels of cyt *b*559 or chlorophyll, we incubated the oxygen-evolving PS II complex with the same amounts of detergent as we used for solubilization, for the same time required to achieve resolution of PS II subcomplexes by sucrose gradient centrifugation, and at the same temperature at which fractionations were made. However, no differences could be observed in the chl/cyt ratios of these samples with respect to a control, indicating that neither DM nor OTG have any side effect.

3.2. *Characterisation of PS II core complexes*

As a first step in the fractionation of oxygen-evolving PS II complexes, we used DM solubilization and density gradient centrifugation. After some trials, we found

Table I

Pigment compositions of Photosystem II complex

chl*a*	pheo*a*	β-carotene	PQ
42	2.0	8	1.5

Values reported are averaged on data from two independent determinations

Table II

Chlorophyll to cytochrome *b*559 ratio in PS II preparations containing different chlorophyll-proteins

PS II complex	PS II core	CP43-depleted PS II core		PS II RC
42 ± 4.0	30 ± 2.1	18 ± 1.5[a]	19 ± 2.2[b]	6 ± 1.5

[a] OTG2 band

[b] 43-depleted PS II core complex obtained as described in [19]

that 10 min solubilization with 0.5% detergent at 0.5 mg chl/ml, after sucrose gradient centrifugation, leads to two well-resolved green bands. The lighter band (DM1), migrating at about 0.3–0.4 M sucrose, contains polypeptides belonging to the chl*a/b* proteins CP29 and CP26 [25] whilst the heavier band (DM2), migrating at about 0.65–0.75 M sucrose, contains only PS II core polypeptides together with small amounts of extrinsic 33-kDa polypeptide (Fig. 1, lane 2). Fractionation of the gradients allowed us to establish, by optical absorption measurements, that about 25–30% of the pigments were located in the DM1 band, whilst 70–75% were found in the DM2 band (Table III). It was also found that cyt *b*559 activity could only be detected in the DM2 band, with a recovery very near to 100% (Table III). A value for the chl/cyt ratio of 30 ± 2 was determined for the DM2 band (Table II), and if we compare this value with that observed in the oxygen-evolving PS II complex, a decrease of 28–29% is found, a value almost identical to the percentage of chlorophyll estimated for the DM1 band on the sucrose gradient. These data strongly suggest that 12–14 pigments are associated with the chl*a/b*-binding proteins, CP29 and CP26.

3.3. *Characterisation of CP43-depleted PS II cores*

As a subsequent step in further PS II fractionation we worked out a method to produce a CP43-depleted PS II preparation. At first we adopted the published method of Akabori et al. [18], based on OTG solubilization and density gradient centrifugation, in the presence of OG. However, this method is known to produce a mixture of CP43-containing and CP43-depleted PS II cores, which are only partially resolved by sucrose gradient centrifugation. After some attempts we found that substitution of OG for OTG in the gradient gave satisfactory results. Using this method two well defined green bands could be resolved, a lighter dark green band (OTG1), containing about 55–60% of total chl but no cyt *b*559 activity, and a heavier band (OTG2) containing about 40–45% of chl and all of the cyt *b*559 activity (Table III). SDS-PAGE of OTG1 showed it contained polypeptides belonging to chl*a/b* proteins and, in addition, CP43 apoproteins, whilst OTG2 contained CP47 apoprotein together with D1, D2 and cyt *b*559 polypeptides (Fig. 1, lane 3). The chl/cyt ratio in the OTG2 band was 18–20 (Table II).

Table III

Distribution of chlorophyll and cyt *b*559 in bands obtained by sucrose gradient centrifugation

	DM1	DM2	OTG1	OTG2
chl	27 ± 2.5	74 ± 5.1	58 ± 4.2	41 ± 3
%cyt	n.d.	97 ± 6	3 ± 1.5	96 ± 4.4

n.d., not detectable

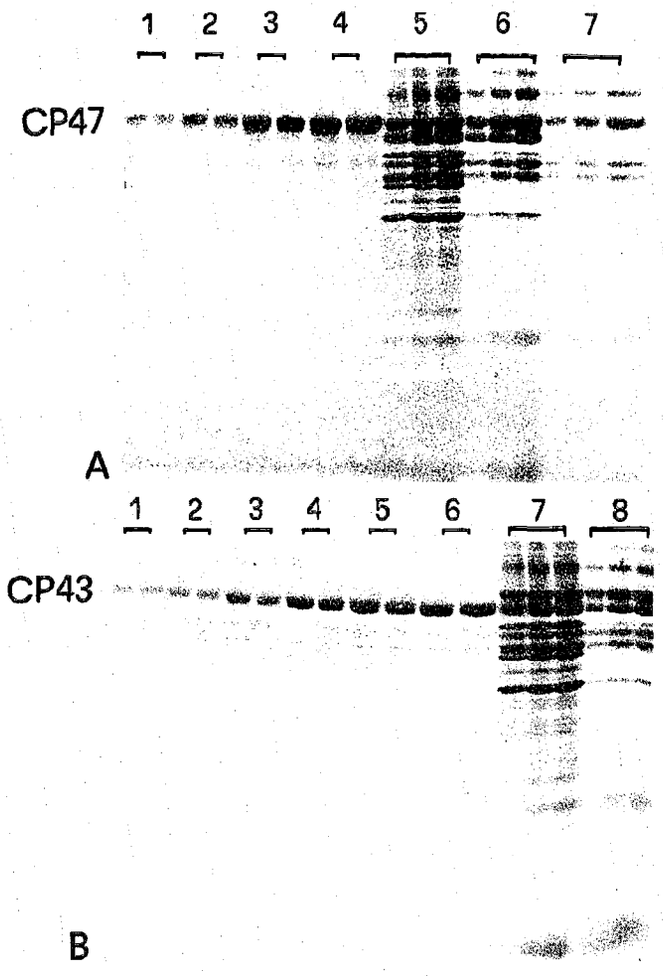
With respect to the chlorophyll level in the oxygen-evolving PS II complex, the decrease of the chl/cyt ratio observed in the OTG2 band is very near, in per cent terms, to the amount of chlorophyll found in the OTG1 band. Thus, if 42 chls are present in the original oxygen-evolving PS II complex, we have to attribute the decrease of the chl/cyt ratio observed in the OTG2 band to the chlorophylls bound to CP26, CP29 and CP43. Because we estimate that 12–14 chls are associated with CP26 and CP29, 10–12 chls should therefore be present in the CP43. We also compared the properties of the OTG2 with those of the better characterized CP43-less PS II complex obtained by anionic exchange chromatography [19]. The polypeptide composition of this complex is shown in Fig. 1, lane 4, and as Table II shows, it contains an equivalent level of chlorophyll as OTG2 band, relative to cyt *b*559.

3.4. *Characterisation of PS II reaction centre complexes*

As a last step in PS II fractionation we isolated PS II RC complexes as described in [19] or in [20]. The polypeptide composition of PS II RC complexes prepared as described in [19] is shown in Fig. 1, lane 5, and we measured its chl/cyt ratio (Table II). In agreement with earlier work [20], values between 5 and 7 were consistently observed with both types of reaction centre preparation, indicating that the chlorophyll level of CP47 should be about 10–13 molecules.

3.5. *Determination of the percent of chlorophyll associated with CP47 and CP43 in PS II complexes and subcomplexes*

Using a complementary approach to the above analyses, we tried to verify our calculation of pigment levels in the different chl-binding proteins of PS II by focusing our attention mainly on CP47 and CP43. We isolated these two complexes (Fig. 1, lanes 6 and 7) in a pure form with pigments still bound. Different amounts of the two different proteins based on chlorophyll levels were subjected to SDS-PAGE (Fig. 2A and B). After Coomassie blue staining, bands were cut from the gels and the dye eluted as described in [22] for determination of optical absorption. The values obtained were plotted against the corresponding amounts of chl and a straight line was found, both for CP47 and CP43 (Fig. 2C). We used these standard curves to estimate, on a dye basis, the percent of pigments associated with CP47 and CP43 in the different PS II preparations. In order to do this the various PS II preparations were loaded onto the same gels (oxygen-evolving PS II complex, lanes 5, Fig. 2A and lanes 7 for Fig. 2B; PS II core complex, lanes 6, Fig. 2A and lanes 8, Fig. 2B; CP43 depleted PS II, lanes 7, Fig. 2A). The values obtained are shown in Table IV and, when compared with those obtained from the chl/cyt ratio (Table III), a close relation is observed between them.



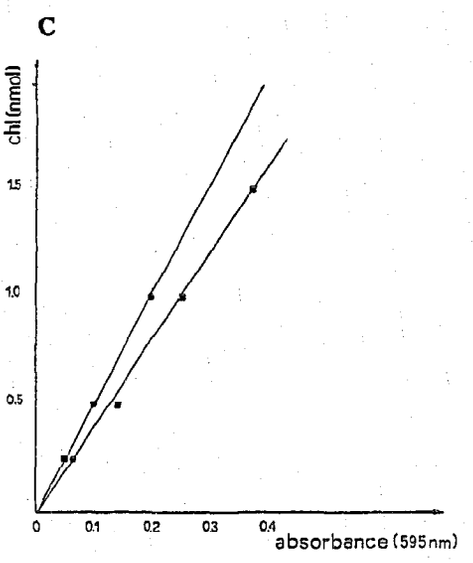


Fig. 2. Data used to estimate the amount of chlorophyll bound to either CP47 or CP43. Gel lanes were loaded with different levels of isolated CP47 in duplicate ((A), lanes 1 to 4) or CP43 ((B), lanes 1 to 6) together with different amounts of PS II preparations. The Coomassie blue eluted from isolated CP47 and CP43 were plotted against the amount of chlorophyll loaded on each lane, to obtain two standard curves (C) where closed circles are CP43 and closed squares CP47. These were used to estimate the amount of chlorophyll associated with CP47 and CP43 in various PS II preparations. Different amounts of oxygen-evolving PS II complex (A) lane 5, and (B) lane 7, PS II cores, (A) lane 6 and (B) lane 8, CP43 depleted PS II, (A) lane 7, were loaded on gels as follows. (A) Lane 1, 0.25 nmol chl CP47; lane 2, 0.5 nmol chl; lane 3, 1.0 nmol chl; lane 4, 1.5 nmol chl; lane 5, PS II complex, 2.0, 4.0 and 5.0 nmol chl respectively; lane 6, PS II core complex, 1.8, 3.6 and 5.4 nmol chl; lane 7, CP43-depleted PS II cores, 0.76, 1.52 and 2.28 nmol chl. (B) Lane 1, 0.25 nmol chl CP43; lane 2, 0.5 nmol chl; lane 3, 1.0 nmol chl; lane 4, 1.5 nmol chl; lane 5, 2.0 nmol chl; lane 6, 2.5 nmol chl; lane 7, PS II complex, 4.0, 6.0 and 8.0 nmol chl; lane 8, PS II core complex, 1.8, 3.4 and 5.4 nmol chl.

Table IV

Percent of chlorophyll associated to CP47 and CP43 in different PS II preparations as deduced from the amount of Coomassie eluted from gel bands shown in Fig. 2. For comparison, the percent of chlorophyll associated to these complexes as deduced on cyt *b*559 base is also reported

	PS II complex		PS II core complex[a]		43-depleted PS II core[b]	
CP47	28.9[c]	25.6[d]	41.6[c]	41.0[d]	55.4	66[d]
CP43	25.0[c]	25.6[d]	44[c]	41.0[d]		

[a] DM2 band;
[b] OTO2 band;
[c] Values calculated from data of Fig. 2;
[d] Values calculated from data in Table III.

4. DISCUSSION

HPLC analyses have been carried out on an oxygen-evolving PS II complex isolated by the procedure of Ghanotakis et al. [11] which is known to contain, besides the core and reaction centre polypeptides, the chl *a*/*b*-binding proteins, CP26 and CP29, but neither CP24 nor LHCII [25,26]. We found that this complex binds 42 chlorophyll molecules in total (chl*a* and chl*b*), 8 β-carotenes and 1.5 plastoquinones, assuming that there are 2 pheophytins per P680. We also found that there was one cytochrome *b*559 per 40 chl in this complex indicating that there is one cyt *b*559 per P680, in

agreement with a recent study on different PS II preparations [27]. Based on this belief we have been able to estimate the total number of chlorophyll molecules associated with isolated PS II cores free of CP26 and CP29. Our estimate of about 30 chls per P680 for PS II cores is similar to that reported by Akabori et al. [18] and only slightly lower than that deduced from analysis of PS II antenna sizes during greening of the barley mutant, chlorina f_2, grown in intermittent light [28,29]. It is, however, significantly smaller than some reports where the level of photoreducible Q_A has been used to estimate antenna sizes of isolated complexes [5,30]. These higher levels could reflect inactivation of some Q_A during the isolation procedure.

Since we estimate the chlorophyll content of the CP43-less complex prepared by two different methods to be about 18 chls then we conclude that CP43 binds 12 chl molecules. A similar number is derived for CP47 bearing-in-mind that the D1/D2/cyt *b*559 RC complex binds 6 chls (Table II and refs [20,31]). The conclusion that CP43 and CP47 bind the same levels of chl is consistent with homologies which exist in their amino acid sequences, especially in conservation of histidine residues, as well as in their predicted secondary structures [12]. It is also consistent with the conclusions of Delepelaire and Chua [13] but these workers estimated that only four to five chlorophylls were bound to each. The reason for these lower values is not clear but could reflect the partial depletion of the proteins of chlorophyll during electrophoresis in the presence of sodium dodecyl sulphate, a detergent which is well known to solubilise bound chlorophylls [32].

The above assignment of the number of chlorophyll molecules to particular PS II complexes and polypeptides is based on the assumption of one cyt *b*559 per P680 or per two pheophytins. If this assumption is incorrect and the amount of cyt *b*559 or pheophytin is higher per P680 then the antenna sizes would be correspondingly larger. Whether or not this is true our conclusions about the relative levels of chlorophyll associated with CP47 and CP43 within PS II were confirmed by Coomassie blue analyses as indicated in Table IV.

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