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FUNCTION OF THE POLYPEPTIDES OF THE PHOTOSYSTEM II REACTION CENTER IN *CHLAMYDOMONAS REINHARDTII*

LOCALIZATION OF THE PRIMARY REACTANTS

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The distribution of the primary quinone and of the pheophytin acceptors has been studied in PS II particles isolated from *Chlamydomonas reinhardtii*, with respect to the distribution of the apoproteins of the two chlorophyll-protein complexes associated with the PS II core. We show that photoreduction of the primary quinone requires the presence of the 50 and 47 kDa polypeptides. On the contrary, charge separation between P-680 and the pheophytin acceptor molecules can occur within the chlorophyll-protein complex of which the 50 kDa polypeptide is the apoprotein. Functional analysis of the PS II fractions shows that an active PS II center contains one photoreducible quinone and one photoreducible pheophytin per 45 chlorophyll molecules. Stoichiometric analysis of the PS II fractions shows that a PS II reaction center contains 45 chlorophyll molecules associated with most likely one copy of the 50 kDa and the 47 kDa polypeptides.

Introduction

Fractionation of the thylakoid membranes of various photosynthetic organisms has led to the characterization of several particles enriched in primary PS II activity [1]. This activity is defined as the photooxidation of a particular chlorophyll, P-680, and the subsequent reduction of a primary acceptor, a plastoquinone-iron complex, Q, via at least one intermediate electron acceptor, a pheophytin molecule I.

In *Chlamydomonas reinhardtii*, the most purified PS II particles which still retain full photochemical activity contain at least five intrinsic membrane polypeotides to which are bound more than 40 chlorophyll molecules [2]. Purified bacterial reaction centers, which contain the same kind of primary electron acceptors as those of PS II, have been shown to be constituted of only three intrinsic membrane polypeptides to which are associated 4 molecules of bacteriochlorophyll and 2 molecules of bacteriopheophytin [3]. The comparatively higher complexity of the PS II particles isolated so far might indicate that the smallest complex containing the PS II center has not yet been isolated from PS II. It was then worth trying to know which polypeptides among the integral membrane polypeptides of the PS II particles were likely to be components of the PS II reaction center.

In *C. reinhardtii*, the intrinsic membrane polypeptides associated with the PS II particles are the following:

Two polypeptides of 50 and 47 kDa, named 5 and 6, respectively, apoproteins of the two chloro-

Abbreviations: PS, photosystem; LHC, light-harvesting complex; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenylcarbazide; DCIP, 2,6-dichlorophenolindophenol.

phyll *a*-protein complexes CP III and CP IV [4], likely equivalents of CPa1 and CPa2 or CP47 and CP43 [5] isolated from higher plants.

Two polypeptides in the 32 kDa range, named D1 and D2 [6], D1 being the so called 'DCMU-binding protein' or 'B-binding protein' [7].

One polypeptide of low molecular weight (less than 10 kDa).

Attempts to correlate the primary PS II activity with the presence of the chlorophyll-protein complex CP47, after isolation of PS II particles from octylglucoside-treated thylakoid membranes from spinach, were recently reported [8,9]. However, these works neither included a quantitative estimation of photoreducible pheophytin nor a direct determination of the photoreduction of Q.

In this paper, we report quantitative and direct measurements of I and Q functionally associated with PS II-active fractions of different polypeptide composition. We have fractionated the PS II-containing band obtained after centrifugation on a sucrose gradient of detergent-solubilized thylakoid membranes from *C. reinhardtii*. For each of these subfractions, we have then measured the distribution of the apoproteins of CPIII and CPIV and the amount of photoreducible I and Q.

Materials and Methods

Two *C. reinhardtii* mutant strains were used in this work: a double-mutant strain, F54.14, devoid of both ATPase complexes and PS II reaction centers [2]; a double-mutant strain, BF4.M18, analogous to the BF4.14 mutant strain previously described [10], lacking PS I reaction centers and largely devoid of PS I peripheral antenna and LHC.

Both mutant strains were grown in Tris-acetate phosphate medium [11] at 25°C under an illumination of 200 lux provided by cool fluorescence lamps.

PS II particle preparation. PS II particles isolated from the F54.14 mutant strain were prepared according to Diner and Wollman [12].

PS II particles isolated from the BF4.M18 mutant strain were prepared in two different ways: (i) a modification of the Diner and Wollman procedure: purified thylakoid membranes, at a final concentration of 0.4 mg Chl/ml, were solubilized at 4°C for 1.5 h in the following medium: 20 mM Mes (pH 5.9)/1 mM EDTA/0.5% Triton X-100/1.25% digitonin. EDTA (1 mM) was also included in the sucrose gradient in order to decrease the contamination of the PS II particles by ATPase complexes; (ii) a modification of the procedure of Mullet et al. [12]: purified thylakoid membranes were washed once in 20 mM Mes (pH 5.9) and then solubilized at room temperature for 20 min in double-distilled water containing 2% Triton X-100 at a chorophyll concentration of 0.4 mg/ml. This suspension was then centrifuged at $50\,000 \times g_{max}$ for 20 min and 8 ml of the supernatant were loaded on a 0.1–1.2 M sucrose gradient (75 ml) containing 0.02% Triton X-100, 1 μM EDTA and overlaid by a 8 ml layer containing 1% Triton X-100, 0.1 M sucrose and 1 μM EDTA. The sucrose gradient was then centrifuged at 40 000 rpm in a 45 Ti rotor on a L5-75 Beckman centrifuge for 15 h at 7°C.

After sucrose-gradient centrifugation, the PS II containing band on the gradient was collected in 0.5-ml fractions corresponding to the different parts of the band.

Q measurements. Q photoreduction in the different PS II subfractions was measured either by the area bounded by the F_{max} and the F_{var} [13] or by the flash-induced absorbance changes at 325 nm [14] using the flash spectrophotometer described by Joliot et al. [15]. In both cases, the experimental conditions were the same: PS II particles (5 μg Chl/ml) were resuspended in 50 mM Hepes (pH 7.5) in the presence of 2 mM $MgCl_2$ and $1 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ and incubated in the dark for 5 min, $5 \cdot 10^{-3}$ M hydroxylamine was then added in the dark, 15 min before the experiment. For the spectrophotometric measurements, a series of 14 short saturating Xenon flashes, spaced 210 ms apart, was given. Absorbance changes at 325 nm were measured using detecting flashes at 7, 50 and 200 ms after each actinic flash. Complementary filters were placed respectively in front of the actinic flash (Corning 9-70) and the photocell (Corning 7-54 plus a 2 M $NiSO_4$ solution, 1.5 cm long). Under our conditions, there is no contribution at 325 nm of Z, the electron donor to P-680. No reoxidation of Q^- occurs in the millisecond time range. However, a 20% increase in the $A_{325\,nm}$ is observed between the first and the sixth actinic flash, as in chloroplasts treated with $1 \cdot 10^{-2}$ M

hydroxylamine. This is due to an incomplete photoreduction of Q after a single saturating flash, because of slow electron donation of hydroxylamine to P-680$^+$ [16]. Absolute concentrations of Q in the different PS II substractions were than taken as the $\Delta A_{325\,nm}$ averaged over the 6th to 14th flash, using an extinction coefficient $\epsilon_{325\,nm}$ of 11.5 $mM^{-1} \cdot cm^{-1}$ [17]. The relative amounts of Q in the different PS II subfractions, as estimated by the area over the fluorescence rise, were similar to those obtained through direct spectrophotometric measurements. This is consistent with a constant yield of charge separation in the different PS II subfractions as viewed by the constant ratio $\Delta A_{325\,nm}$ 1st flash/$\Delta A_{325\,nm}$ 6th flash.

I measurements. The photoactive pheophytin was measured spectrophotometrically, by accumulation in continuous light of I^- under strongly reducing conditions. PS II gradient fractions were resuspended in 50 mM Hepes (pH 7.5) at 3 μg Chl/ml in the presence of the following redox mediators: 10 μM pyocyanin, 10 μM phenazine methosulfate, 20 μM 1,2-naphthoquinone, 20 μM 1,4-naphthoquinone, 20 μM duroquinone and 20 μM antraquinone disulfonate. A redox potential of approx. -470 mV was maintained throughout the experiment by the addition of 2 mM dithionite under anaerobic conditions (argon flow). In these conditions, a preillumination of 10 min in continuous strong light provided by a 2000 W lamp filtered through a Wratten No. 16 was given which, on one hand, irreversibly bleaches some chlorophyll and, on the other hand, reduces pheophytin reversibly. After dark adaptation for 20 min, during which reduced pheophytin is reoxidized, the particles were illuminated in continuous strong light and light-induced absorbance changes were measured at 425 nm, a negative peak of the $I^- - I$ difference spectrum [18], during this continuous illumination. The photoreduction of I was found to be fully reversible after an incubation in the dark of 20 min. This light-induced absorbance change is shown Fig. 1A, upper curve: the kinetics are biphasic with the slow phase of constant slope due to chlorophyll bleaching. To eliminate the contribution of this chlorophyll bleaching, a second illumination was performed on the particles and the light-induced absorbance changes were measured at 450 nm, a positive peak of the $I^- - I$ difference spectrum (Fig. 1A, lower curve). At this wavelength, the chlorophyll bleaching appears to be of the same sign and amplitude as at 425 nm. The difference $\Delta A_{425\,nm} - \Delta A_{450\,nm}$ was then calculated (Fig. 1B), in which is only present the contribution of $I - I^-$. Absolute amounts of I were then calculated, assuming an extinction coefficient $\epsilon_{425-450\,nm}$ of 54 $mM^{-1} \cdot cm^{-1}$ [18].

Chlorophyll to protein ratio. Chlorophyll to protein ratios were measured in PS II particles isolated from the BF4.M18 double mutant, assuming

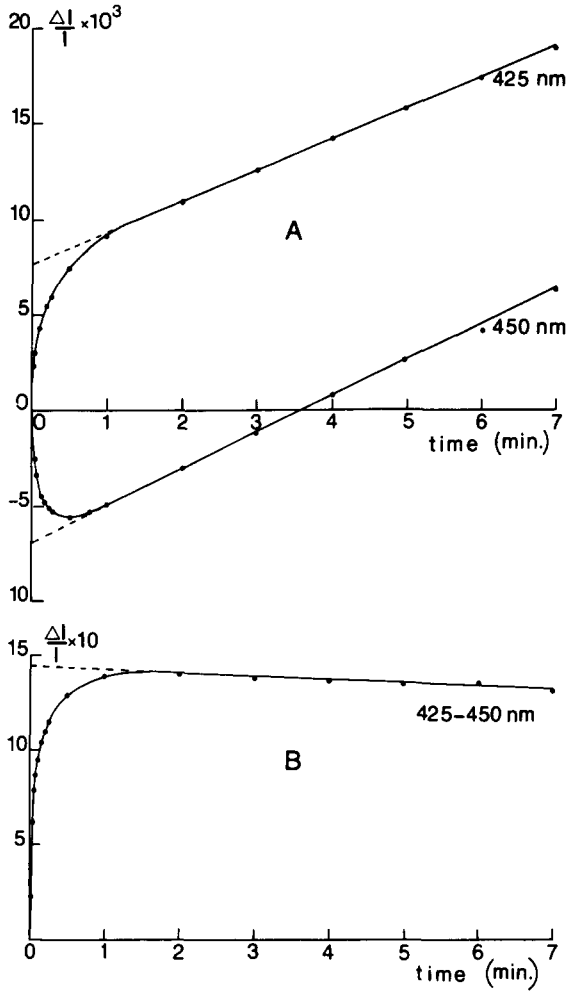

Fig. 1. (A) Light-induced absorbance changes in PS II particles of F54.14 at -470 mV measured at 425 and 450 nm as a function of illumination time. (B) Absorbance change resulting from the difference between $\Delta_{425\,nm}$ and $\Delta A_{450\,nm}$. For details, see the text.

that polypeptides 5 and 6 were the sole chlorophyll-binding polypeptides in the PS II particles. To this end, absolute amounts of polypeptides 5 and 6 were measured for a given amount of chlorophyll in the PS II particles: a small number of BF4.M18 cells were inoculated in a 2-l Erlenmeyer flask containing Tris-acetate/phosphate medium to which was added [^{14}C]acetate (54 Ci/mmol, 1 μCi/ml). The cells were then grown up to stationary phase ($6 \cdot 10^6$ cells/ml) so as to reach the isotopic dilution of the medium. PS II particles were then isolated following the method described above (Triton-digitonin treatment). Labeled particles corresponding to a known amount of chlorophyll were then analyzed on a 7.5–10% polyacrylamide gradient gel. After electrophoresis, the two bands corresponding to polypeptides 5 and 6 were excised from the gel, dissolved in 0.6 ml of a perchloric acid/H_2O_2 mixture and counted after addition of 10 ml Aquasol. Knowing the isotopic dilution of ^{14}C in the culture medium, the specific activity, the counting efficiency, the molecular weight of each polypeptide (respectively 50 and 47 kDa) and the percent of carbon in these polypeptides (taken as 53% from calculated values for LHC polypeptides [20]), we were then able to determine the absolute amounts of polypeptides 5 and 6 and thus the chlorophyll to protein ratio. Chlorophyll concentrations were measured according to Arnon [19].

Gel electrophoresis and polypeptide analysis. Polypeptides were analyzed by gel electrophoresis which was carried out according to Laemmli [21] using 7.5–15% polyacrylamide gradient gels. Samples for electrophoresis were prepared in the presence of 2% SDS, 50 mM dithiothreitol-Na_2CO_3, 12% sucrose and they were heated at 100°C for 50 s before electrophoresis. Relative amounts of the 50 and 47 kDa polypeptides were measured on the densitometric scans of the Coomassie blue-stained gels according to De Vitry et al. [22].

Results

We first have prepared PS II particles totally devoid of LHC contamination by the use of the double mutant strain BF4.M18. Fractions of the PS II band on the sucrose gradient loaded with Triton-digitonin-solubilized thylakoid membranes were analyzed by gel electrophoresis (Fig. 2). Besides the intrinsic membrane polypeptides 5 and 6 (50 and 47 kDa), respectively associated with the chlorophyll-protein complexes III and IV, they contain: polypeptides 12 and 24 (30 and 18 kDa), which are extrinsic and not essential to the primary photochemical activity [23]; two intrinsic membrane polypeptides, 27 and 36 (15 and 3 kDa). 27 is not essential to the primary activity since it is not always found in the particles. 36 is likely to be associated with cytochrome *b*-559 [23]. The intrinsic membrane polypeptide D2 (approx. 32 kDa) is clearly visible at the front of polypeptide 12, contrary to D1 which is not stained with Coomassie brilliant blue, although it is probably present in these particles as it is the case in ^{14}C-labeled PS II particles isolated from the WT strain of *C. reinhardtii* [24]. The minor bands in Fig. 2 are present in substoichiometric amounts as compared to the main polypeptides and are considered as contaminants of the PS II preparation.

Thus, only polypeptides 5, 6, D1 and D2 are likely to be associated with the primary PS II photoreactants. The molar fractions of polypeptides 5 and 6 (MF5 = 5/5 + 6 and MF6 = 6/5

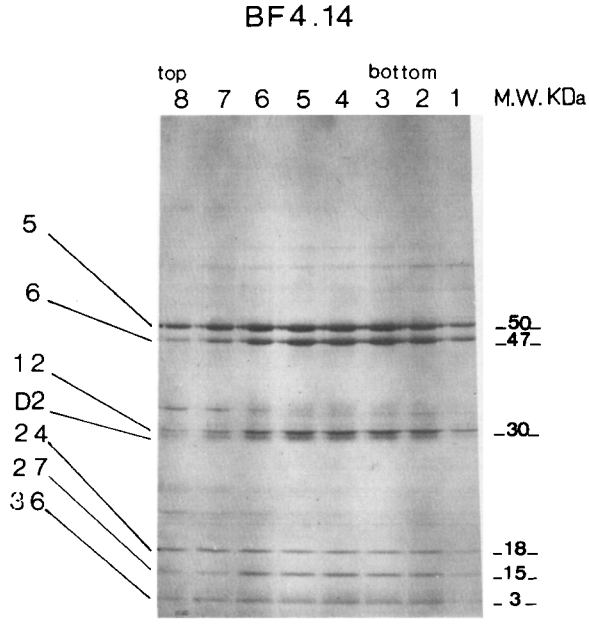

Fig. 2. Polypeptide pattern of PS II fractions from the BF4.M18 mutant strain. On the left is indicated the nomenclature of the polypeptides and on the right their apparent molecular weight.

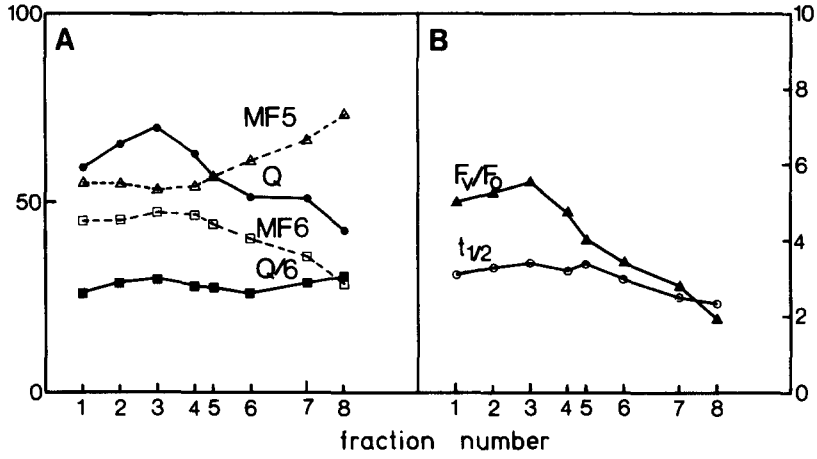

Fig. 3. (A) Distribution of MF5, MF6, Q/Chl and Q/Chl·MF6 among the PS II fractions of the BF4.M18 mutant. Q was measured by the area over the fluorescence rise. (B) Distribution of F_v/F_o and $t_{1/2}$ among the PS II fractions of the BF4.M18 mutant. All parameters are given in arbitrary units except MF5, MF6 and F_v/F_o.

+ 6) are roughly equal in the lower part of the band and MF5/MF6 increases towards the upper part of the band up to 2.6 (Fig. 3A). The F_v/F_o ratios (Fig. 3B) and the amount of Q on a chlorophyll basis (Fig. 3A) are maximal when the MF5/MF6 ratio is close to 1. Furthermore, in this gradient, the PS II activity (amount of Q on a chlorophyll basis) is strictly correlated to MF6 as shown Fig. 3A (see Q/MF6). One also observes (Fig. 3B) that the half-time for the fluorescence induction curves stays about the same in all the fractions, indicating that the PS II complexes able to carry out Q photoreduction contain a constant number of associated antenna chlorophylls.

We then analyzed the subfractions of the PS II band isolated from the F54.14 double mutant having a MF5/MF6 ratio smaller than 1 (see Fig. 4). The MF5/MF6 ratio increases from the bottom to the top of the PS II band from 0.43 to 1. In the LHC uncontaminated part (fractions 1–5) we observe: an increase of the MF5/MF6 ratio from 0.43 to 0.88, an increase of the F_v/F_o ratio from

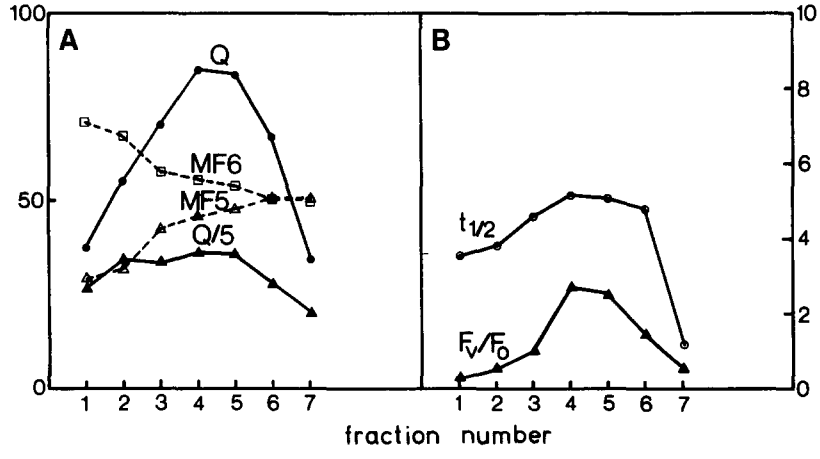

Fig. 4. (A) Distribution of MF5, MF6, Q/Chl and Q/Chl·MF5 among the PS II fraction of the F54.14 mutant. (B) Distribution of F_v/F_o and $t_{1/2}$ among the PS II fractions of the F54.14 mutant.

TABLE I

PS II CONTENT IN POLYPEPTIDE 5 AND 6 AND IN Q AND I ACCEPTOR MOLECULES IN THREE DIFFERENT PS II SUBFRACTIONS

F54.14M: analogous to fraction 4 (see Fig. 4A). BF4.M18M, BF4.M18H: fractions isolated from the middle (M) and upper (H) part of the PS II band from the BF4.M18, after Triton treatment at room temperature. Chl/Q, Chl/I, I/Q and 5/6 are expressed as molar ratios, whereas Q/MF5, Q/MF6, I/MF5 and I/MF6 are in arbitrary units, normalized to Q/MF6 in F54.14M. I and Q were measured spectrophotometrically.

	Chl/Q	Chl/I	I/Q	5/6	Q/MF5	Q/MF6	I/MF5	I/MF6
F54.14M	46	42	1.1	1.1	0.9	1	1	1.1
BF4.M18M	49	44	1.1	1.4	0.75	1.1	0.86	1.2
BF4.M18H	98	30	3.3	3	0.28	0.9	0.94	2.9

0.2 to 2.5, an increase of the amount of Q/Chl, a slight increase of the half-time of the fluorescence induction curve and a constant value of the Q/MF5 ratio. In the upper part of the PS II band (fractions 6–7), the Q/MF5 ratio and the half-time of the fluorescence induction decrease because of some contamination by the LHC. The concomitant decrease in $t_{1/2}$ and F_v/F_o ratio in this part of the PS II band indicate that the LHC present in these PS II subfractions is functionally associated with them, although the energy transfer efficiency between the two is low.

From these experiments (Figs. 2–4) we concluded that the primary PS II activity, as measured by the photoreduction of Q, depends on the presence of both polypeptides 5 and 6 in a 1 : 1 molar ratio. This does not preclude the localization of any of the primary reactants on one or the other of these polypeptides. To this end, we have measured light-induced absorption changes associated with Q or I reduction in three PS II subfractions having different polypeptide 5/polypeptide 6 molar ratios (see Table I). Three main observations can be drawn from this table:

– The Q/MF6 ratio is nearly constant in the three fractions: this is consistent with the results in Fig. 3A since MF6 is smaller than MF5 in these three fractions.

– The I/MF5 ratio is nearly constant in the three fractions.

– The I/Q ratio is close to 1 in the fractions where the MF5/MF6 ratio is also close to 1, whereas it increases up to 3.3 in the fraction having a MF5/MF6 ratio of 3.

These two last observations indicate that the charge separation between P-680 and I still occurs in the absence of polypeptide 6, whereas Q photoreduction can only occur in those fractions having both polypeptides 5 and 6 in a 1 : 1 molar ratio. P-680 and I are then most likely bound to polypeptide 5.

Direct measurement of the chlorophyll to protein ratio using ^{14}C-labeled particles gives a value of 23 ± 2 chlorophyll molecules per polypeptide chain, if one assumes an equal distribution of chlorophyll among both polypeptides 5 and 6. If we consider that Q-photoactive PS II centers contain one copy of each polypeptide 5 and 6 and that I-photoactive centers contain one copy of polypeptide 5, the calculated chlorophyll/polypeptide 5 or 6 ratio for the three PS II subfractions varies from 20 to 26 (F54.14M: 22 ± 2, BF4.M18M: 23 ± 3, BF4.M18H: 24 ± 1) and fits with the direct measurement. This comparison shows that the fraction of inactive PS II particles is negligible in our preparation and that the core antenna surrounding the PS II reaction center contains about 45 chlorophyll molecules and consequently a single copy of each polypeptide 5 and 6.

Discussion

According to Okamura and Feher [3], a purified reaction center is the smallest possible entity still able to carry out primary photochemistry with unchanged back-reaction kinetics at cryogenic temperature as compared to intact membrane.

The PS II preparations purified from Triton-digitonin-solubilized thylakoid membrane from *C. reinhardtii* show the same back-reaction properties

as in intact membranes [32], and are consequently a good starting material to further characterize the essential components of the PS II reaction center, although they may still contain some contaminants.

In the present work, we have shown that a PS II reaction center complex, isolated from *C. reinhardtii* contains together with 45 chlorophyll molecules bound to two polypeptide chains of respectively 50 and 47 kDa one primary electron donor (P-680), one intermediate electron acceptor (I) and the first stable electron acceptor (Q). The possible involvement of other components will be discussed later.

This result does not support our earlier model in which the PS II center was made of an heterodimer of both polypeptides of 50 and 47 kDa [22]. This was based on two assumptions which might be incorrect: a dimeric structure in the PS I reaction center [25] and an equal contribution of all PS I and PS II reaction centers to the electric field.

From a comparison of the half-times in the fluorescence rise of intact chloroplasts and PS II reaction center preparations, we calculate that the size of a PS II unit is 6.8-times larger than that of the PS II core, i.e., 313 ± 27 chlorophylls vs. 46 ± 4 chlorophylls. The size of the PS II core antenna correlates well with what was obtained on *Cyanidium caldarium* [26].

Our observation of charge separation between P-680 and I still occurring in the absence of polypeptide 6 is consistent with the observation by Nakatani et al. [8], who reported that fluorescence quenching due to pheophytin photoreduction occurs in purified CP47 (analogous to CPIII) isolated by electrophoresis of octylglucoside-treated thylakoid membranes. However, at variance with others [8,27], we could not detect any enrichment in the 695 nm fluorescence emission at 77 K in PS II particles having a high I/Q ratio and the 77 K fluorescence emission spectra always peaked at 685 nm with a rather small shoulder at 695 nm (not shown). Other parameters (detergent used, presence of orthophenanthroline [28]) could modify the fluorescence characteristics of the particles.

An excess of either one of the two polypeptides (47 or 50 kDa) decreases both the amount of photoreduced Q and the F_v/F_o ratio, indicative of chlorophyll not connected to an active center. These observations are in contradiction with data reported by Camm and Green [9] on PS II fractions isolated after octylglucoside treatment of spinach thylakoid membranes. These authors conclude that the PS II activity in their subfractions is proportional to the sole presence of CPa1 (equivalent to the CPIII of *C. reinhardtii*, i.e., polypeptide 5) based on the two following measurements:

(i) DPC–DCIP electron transfer in each subfraction: this indirect measurement of the primary quinone reduction is not as reliable as light-induced absorbance changes since it assumes that the rate of electron transfer from Q to DCIP is high (to compete with the back-reaction) and the same for all the subfractions. Our PS II particles show high specific activity as measured by light-induced asorbance changes but poor electron transfer rates from DPC to DCIP (Diner, B., personal communication).

(ii) Scanning of the chlorophyll bound to CPa1 in each subfraction on gels run at 4°C: the stability of CPa1 and CPa2 could be differential and vary from one fraction to another upon gel electrophoresis, leading to over or under-estimation of their relative amounts. That a PS II reaction center is in an active state only when both polypeptides 5 and 6 are associated in a 1 : 1 molar ratio does not preclude the possibility that the binding site of the primary quinone is on one polypeptide only; the association of polypeptides of 5 and 6 would bring about the correct positioning of the primary reactants. It is the case in the bacterial reaction center in which the primary acceptor Q_A has been located by photoaffinity labeling with an azidoquinone on the M subunit but in which the presence of L and M subunits is necessary to observe Q_A photoreduction [31].

In our work, we have not closely analyzed the distribution of the two main other intrinsic membrane polypeptides associated with the PS II subfractions, D1 and D2, although they could play a role in the primary PS II photochemistry. Up to now, there is no evidence of chlorophyll-binding sites on these polypeptides. However, D1, as subunit L in the bacterial reaction center, has been shown to bind azidoatrazin [7,29] and there are sequence homologies between D1 and D2 (Rochaix, J.D., personal communication) as well as between D1 and the L and M subunits of the

bacterial reaction center [30]. Due to the lack of correlation between polypeptides 5 and D2 in our gradient (see Fig. 2), we feel confident that the location of P-680 and I on polypeptide 5 is clearly established. However, besides the requirement of both polypeptides 5 and 6 for primary PS II photochemistry, the possible involvement of polypeptides D1 and D2 in quinone-binding sites requires further investigation.

Acknowledgements

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