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CHARACTERIZATION OF A RESOLVED OXYGEN-EVOLVING PHOTOSYSTEM II PREPARATION FROM SPINACH THYLAKOIDS

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An oxygen-evolving Photosystem (PS) II preparation was isolated after Triton X-100 treatment of spinach thylakoids in the presence of Mg^{2+}. The structural and functional components of this preparation have been identified by SDS-polyacrylamide gel electrophoresis and sensitive spectrophotometric analysis. The main findings were: (1) The concentration of the primary acceptor Q of PS II was 1 per 230 chlorophyll molecules. (2) There are 6 to 7 plastoquinone molecules associated with a 'quinone-pool' reducible by Q. (3) The only cytochrome present in significant amounts (cytochrome *b*-559) occurred at a concentration of 1 per 125 chlorophyll molecules. (4) The only kind of photochemical reaction center complex present was identified by fluorescence induction kinetic analysis as PS II_α. (5) An $E_m = -10$ mV has been measured at pH 7.8 for the primary electron acceptor Q_α of PS II_α. (6) With conventional SDS-polyacrylamide gel electrophoresis, the preparation was resolved into 13 prominent polypeptide bands with relative molecular masses of 63, 55, 51, 48, 37, 33, 28, 27, 25, 22, 15, 13 and 10 kDa. The 28 kDa band was identified as the PS II light-harvesting chlorophyll *a*/*b*-protein. In the presence of 2 M urea, however, SDS-polyacrylamide gel electrophoresis showed seven prominent polypeptides with molecular masses of 47, 39, 31, 29, 27, 26 and 13 kDa as well as several minor components. CP I under identical conditions had a molecular mass of 60–63 kDa.

Introduction

In higher plant chloroplasts, the light-induced charge separation at PS II provides the electrons for the reduction of plastoquinone and the positive charges for the oxidation of water. Although significant advances have recently been made in understanding the mechanism of charge separation in PS II [1–5], the reactions involved in oxygen evolution, as well as questions concerning the function of the primary electron acceptor Q of PS II, remain to be solved. In addition, many fundamental questions concerning the molecular composition and organization of the PS II complex remain unanswered. Attempts to provide answers to the above questions have involved work with mutants which are devoid of PS I [6], and also with isolated active PS II preparations from chloroplast thylakoids [7–11]. Many of the previously described PS II preparations, however, had lost their ability to evolve oxygen (see Ref. 11), and although useful for characterization of primary reactions, they could not provide information on

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Abbreviations: Chl, chlorophyll; PS, photosystem; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CP, chlorophyll-protein complex; PQ, plastoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

secondary electron-transfer reactions. In addition, several of these PS II preparations were contaminated with other non PS II-related complexes [11]. Recently, Berthold et al. [12] have described a method for the isolation of an oxygen-evolving PS II preparation which appears to be devoid of PS I as well as the chloroplast cytochrome *b-f* complex. Previous work from our laboratory has demonstrated that this PS II preparation is active in the reconstitution of electron-transport pathways that are known to occur in unfractionated membranes [13,14]. Apparently, the PS II preparation of Berthold et al. [12] retains a great deal of the structural and functional integrity of the native PS II complex. To this date, however, no detailed analysis has yet been reported on the structural and functional composition of this preparation. In the present work, we applied sensitive spectrophotometric and fluorometric analysis as well as SDS-polyacrylamide gel electrophoresis in the elucidation of the structural and functional organization of the components in the PS II preparation. We discuss our results in terms of PS II function in vivo.

Materials and Methods

An oxygen-evolving PS II preparation was isolated by slight modification of the procedure of Berthold et al. [12]. Intact chloroplasts were prepared from freshly harvested greenhouse spinach by blending leaves in a solution containing 0.4 M sucrose, 50 mM Tris-HCl buffer (pH 7.8), 10 mM NaCl. The resulting slurry was passed through filtering silk and the filtrate centrifuged at $3000 \times g$ for 1 min to pellet intact chloroplasts. The chloroplasts were resuspended in a solution containing 20 mM Hepes buffer (pH 7.0), 5 mM $MgCl_2$ and 15 mM NaCl. Treatment of this suspension with Triton was done according to the procedure of Berthold et al. [12]. The final PS II-containing pellet was resuspended in 0.3 M sucrose, 20 mM Hepes buffer (pH 7.0), 5 mM $MgCl_2$ and 15 mM NaCl. The preparation was stored at 77 K in small aliquots. This preparation gave rates of O_2 evolution of approx. 100 μmol/mg Chl per h when assayed with 3 mM potassium ferricyanide as the electron acceptor. Oxygen evolution was measured with a Rank electrode; samples were illuminated with light passing through a 530 nm cutoff filter (Corning CS 3-68).

Absorbance spectra were recorded in either an Aminco DW-2a or a Cary 219 recording spectrophotometer. Light-induced absorbance changes of the reaction center chlorophyll of PS I (P-700) and the primary electron acceptor of PS II (Q) were measured at 700 and 320 nm, respectively, in a sensitive split-beam spectrophotometer [15,16]. Extinction coefficients of 64 and 13 $mM^{-1} \cdot cm^{-1}$ were used, respectively, for all calculations. The same instrument was used for analysis of light-induced fluorescence changes. Chemical difference spectra of cytochromes were measured in an Aminco DW-2a spectrophotometer operating in the split-beam mode. EPR spectra at cryogenic temperatures were measured in a modified JEOL X-band spectrometer operating with 100 kHz field modulation [17].

Redox titrations of the PS II fluorescence quencher were done using methods previously described [18]. Fluorescence was excited in the blue region and measured in the red region using a chopped measuring beam. The redox cuvette was as described by Dutton [19] and the specific redox mediators used are described in the figure legends. Reductive titrations were done with 0.1 M sodium dithionite in 0.09 M NaOH and oxidative titrations with 5 mM potassium ferricyanide.

SDS-polyacrylamide gel electrophoresis analysis of the PS II preparation was done in a 10–15% linear acrylamide gradient slab gel as described by Chua [20]. When indicated, 2 M urea was included in all steps. Samples containing approx. 10 μg Chl were analyzed. Gels were run for 12–14 h at 4°C with a current of 7 mA. Staining and subsequent treatment were as described in Ref. 20. Standards used were: bovine serum albumin, ovalbumin, trypsinogen, trypsin inhibitor and cytochrome *c*.

Results

Spectroscopic characterization of components in the PS II preparation

The Chl *a*/Chl *b* ratio of the PS II preparation was about 1.6 as compared to a ratio of approx. 3.0 in unfractionated thylakoid membranes. This

suggests the preparation is enriched in the PS II-Chl *a/b* light-harvesting chlorophyll-protein (LHCP). The presence of this component has been confirmed by SDS-polyacrylamide gel electrophoresis analysis of the preparation (see below).

The concentration of Q, the primary electron acceptor of PS II, has been estimated on the basis of the light-induced absorbance increase at 320 nm originating from plastosemiquinone anion formation [21]. A value of 1 Q per 230 Chl has been found in the PS II preparation while values of 1 Q per 300–350 Chl have been reported in unfractionated thylakoids by a similar method [22]. Thus, the PS II preparation shows a 30–50% enrichment in the PS II reaction center based on this measurement. It is worth noting that the isolation procedure used consistently gave a 35–40% yield of total chlorophyll, which indicates an overall recovery of approx. 50–60% of the total PS II reaction centers.

The total plastoquinone content of the preparation was estimated by two different methods: chemical extraction in petroleum ether and then spectrophotometric determination in the ultraviolet from the oxidized-minus-reduced difference spectra [23]; and from the analysis of the area over the fluorescence induction curve in the presence and absence of DCMU [24]. In the latter case, the fluorescence maximum (F_m) of the induction curve in the presence and absence of DCMU was normalized to give the same value. The ratio of PQ/Chl obtained by these two independent methods indicated approx. 1 PQ per 35–40 Chl or a PQ/Q ratio of 6–7. It has been previously reported in unfractionated thylakoids that the secondary electron acceptor pool can accept approx. 14 electrons [24,25], and our fluorescence analysis of the PS II preparation has indicated that the plastoquinone pool can accept 12–14 electrons [14]. Thus, it appears that the PS II preparation retains a ratio of Q to photoreducible plastoquinone that is comparable to that detected in unfractionated membranes. The photoreduction of plastoquinone was DCMU-sensitive, suggesting the preservation of the DCMU-binding site between Q and the plastoquinone site in this PS II preparation.

The P-700 content of the PS II preparation has been estimated from the light-induced absorbance change at 700 nm using a sensitive spectrophotometric procedure [15,16]. This system can detect as little as 5 nmol P-700 at a chlorophyll concentration of 250 μM, which corresponds to a P-700 concentration of 1 per 50 000 Chl. By this method, the PS II preparation was found to contain less than 1 P-700 per 20 000 Chl, and this finding, as well as additional results described below, confirms the absence of any significant amount of PS I in this preparation. Berthold et al. [12] also reported the absence of P-700 based on EPR measurements at 25°C.

Berthold et al. [12] have reported that the only cytochrome detected in the PS II preparation is cytochrome *b*-559. The reported absence of cytochrome b_6 and *f* indicates that the preparation is free of the electron-transfer complex which contains these carriers as well as the Rieske iron-sulfur center [26]. However, if one treats the PS II preparation with 0.2% Triton, all the cytochrome *b*-559 is converted to the low-potential form and a small amount of cytochrome *f* can be detected by the hydroquinone-minus-ferricyanide difference spectrum. Without the Triton treatment, this absorbance is probably masked by the much larger cytochrome *b*-559 absorbance change. It was not possible to determine the concentration, if any, of cytochrome b_6 in the PS II preparation. We have determined the cytochrome *b*-559 concentration is about 1 cytochrome per 125 Chl or about 2 per reaction center, based on the concentration of Q. This value is similar to that obtained in unfractionated chloroplasts [27,28]. Fig. 1 shows the chemical reduced-minus-oxidized difference spectra in the cytochrome α- and β-band region. Approx. 80% of the cytochrome in the preparation is in the oxidized form and can be fully reduced after the addition of dithionite. A portion of the cytochrome (approx. 20%) appears to be in a high-potential form which is reducible by hydroquinone. At pH 6.0, under conditions of oxygen evolution in steady-state light with or without ferricyanide present as an electron acceptor, no significant light-induced absorbance changes of cytochrome *b*-559 (560–570 nm) were observed. However, after addition of ascorbate and incubation in the dark, some photooxidation of this cytochrome is observed.

EPR analysis of the PS II preparation in the

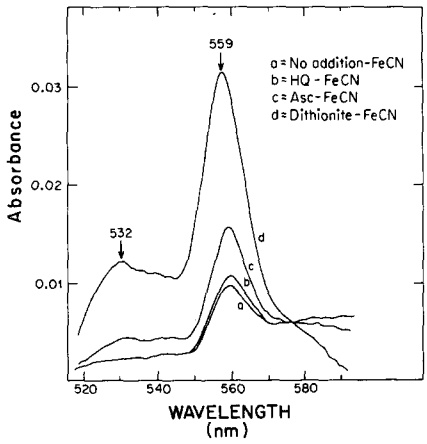

Fig. 1. Chemical difference spectra of cytochromes in an O_2-evolving PS II preparation. The PS II preparation was suspended in 80 mM sucrose, 20 mM Mes (pH 6.0), 5 mM $MgCl_2$ and 15 mM NaCl at a chlorophyll concentration of 0.16 mg Chl/ml. Potassium ferricyanide (FeCN), hydroquinone (HQ) sodium ascorbate (Asc) and sodium dithionite were added in small quantities of the solid form as indicated. The difference absorbance spectra were then taken. The various spectra were balanced at 575 nm.

$g = 2.00$ region indicates the presence of a signal from the component associated with Signal II [29]. Previous work has identified this component as an electron donor to the PS II reaction center chlorophyll, P-680 [30,31]. As shown in Fig. 2, in the presence of ascorbate, the light-induced change in the $g = 2.00$ region after illumination at 15 K is small. Under these conditions, P-700, if present, would undergo photooxidation to P-700$^+$ with a characteristic light-induced EPR signal appearing [29,32]. The results of Fig. 2 confirm independently the negligible amount of P-700 in this preparation. After the addition of ferricyanide (Fig. 2), illumination at 15 K induced a large signal in the $g = 2.00$ region which originates from the photooxidized form of Signal II as well as from the photooxidation of a component with a narrower free-radical signal. The light-induced signals observed after illumination in the presence of ferricyanide are stable in the dark and show no significant decay over a period of 10 min or longer.

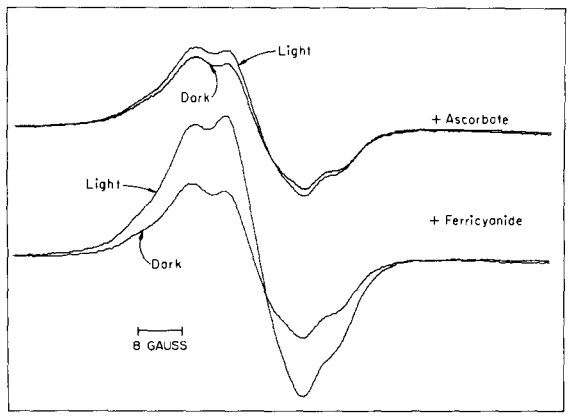

Fig. 2. EPR spectra of an O_2-evolving PS II prepartion. EPR spectra were recorded at 15 K at a chlorophyll concentration of 0.5 mg/ml. Where present, ascorbate and ferricyanide were added at concentrations of 1 mM. Samples were illuminated for 30 s at 15 K with light from a 150 W quartz-tungsten lamp passing through a Corning 2-64 filter. EPR conditions: 3280 ± 50 G; 2.0 G modulation amplitude; 0.02 mW microwave power.

TABLE I

COMPOSITION OF AN O_2-EVOLVING PS II PREPARATION

Chl *a*/Chl *b*	1.6 ± 0.2
Q/Chl	1/230 ± 20
PQ/Chl (chemical)	1/35 ± 5
PQ/Chl (photochemical)	1/40 ± 5
PQ/Q	6 ± 1
Cytochrome *b*-559/Chl	1/125 ± 5
Cytochrome *b*-559/Q	2.1 ± 0.2
Cytochrome *b*-563/Chl	not detected
Cytochrome *f*/Chl	≤ 1/2700
P-700/Chl	≤ 1/20000

A summary of the composition of the PS II preparation is given in Table I.

Fluorescence studies with the PS II preparation

The kinetics of the fluorescence induction in the PS II preparation have been analyzed as described by Melis and Homann [33] in the presence of tetraphenylboron and DCMU. The fluorescence induction curve is shown in Fig. 3A and a semilogarithmic plot of the area over the variable fluorescence is shown in Fig. 3B. This kinetic analysis revealed a monophasic non-first-order process in the photoconversion of Q. A rate constant of 9 s^{-1} was estimated from the slope at zero time (see Fig. 3B). The value of the rate constant (slope in Fig. 3B) increased with time, suggesting the organization of the PS II units in aggregates of up to two

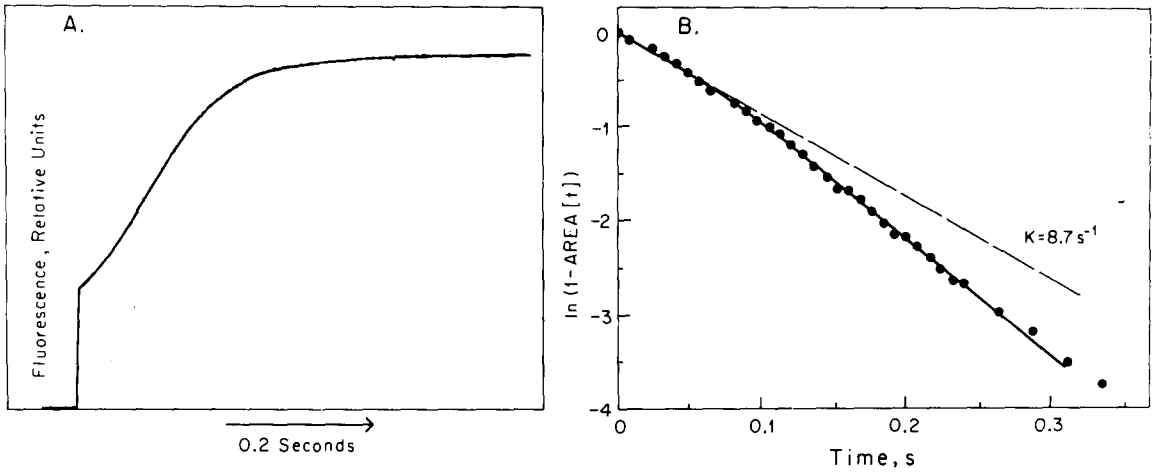

Fig. 3. Fluorescence induction kinetics of Q in an O_2-evolving PS II preparation. The PS II preparation (0.1 mg Chl/ml) was suspended in 50 mM Tricine (pH 7.8), 400 mM sucrose, 10 mM NaCl and 5 mM $MgCl_2$. In addition, 25 μM DCMU and 10 μM tetraphenylboron were also present. The actual fluorescence trace (A) and the kinetic analysis (B) of the area over the variable portion of the fluorescence increase are shown.

units. Working with unfractionated chloroplasts under identical illumination conditions, we measured a biphasic fluorescence induction curve with rate constants $K_\alpha = 10$ and $K_\beta = 5\ s^{-1}$, reflecting the photoactivity of two types of PS II, PS II_α and PS II_β [33–35]. The lack of a β-phase in our Triton-PS II preparation indicates the selective elimination of PS II_β. The deviation of the semilogarithmic plot from linearity and the sigmoidicity of the fluorescence curve (Fig. 3A and B) suggest that the kinetics reflect the photoactivity of PS II_α only. The slower (about 10%) initial rate of PS II_α in the Triton-PS II preparation suggests that a small portion of the chlorophyll antenna has been disconnected from the reaction center P-680 during the treatment, a claim supported by the relatively higher yield of the nonvariable fluorescence emission F_0.

A redox titration of the photochemical fluorescence quencher in PS II_α has been attempted in our preparation (Fig. 4). In this titration we initially used the same redox mediators previously used by ourselves [18] and by others [36–38] in titrations of Q. In the reductive direction, the major transition occurred with an $E_m \approx +150$ mV, but a large hysteresis effect was observed in that this titration was not reversible in the oxidative direction. This result was taken as an indication of poor equilibration between the added redox mediators and Q in the PS II preparation. To overcome this problem, different mediator combinations were used in order to obtain more reversible titrations

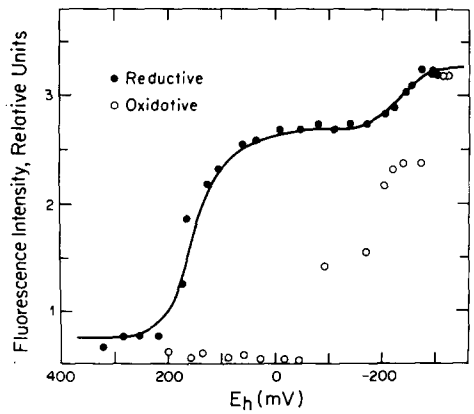

Fig. 4. Redox titration of Q in an O_2-evolving PS II preparation in the presence of quinone-type mediators. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.8), 10 mM $MgCl_2$ and the PS II preparation at a chlorophyll concentration of 15 μg/ml. The following redox mediators were present at a concentration of 10 μM: 1,2-naphthoquinone, 1,4-naphthoquinone, duroquinone, 2,5-dihydroxybenzoquinone, 2-hydroxynaphthoquinone, anthraquinonedisulfonate, anthraquinonesulfonate and safranin O. The redox potential was adjusted to approx. +400 mV with ferricyanide and a reductive titration done using 0.1 M sodium dithionite in 0.09 M NaOH. Titration in the oxidative direction was then done with ferricyanide. Titrations were done in an argon atmosphere as described in Materials and Methods.

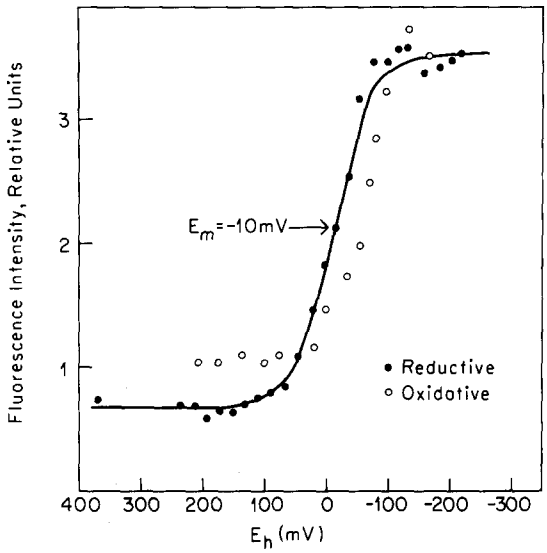

Fig. 5. Redox titration of Q in an O_2-evolving PS II preparation in the absence of quinone-type mediators. The reaction mixture and conditions were as in Fig. 4 except that the following redox mediators (10 μM final concentration) were used instead: phenazine methosulfate, pyocyanine, indigodisulfonate, alloxazine, phenosafranine and acridine.

and, as shown in Fig. 5, these results show a single redox transition with an $E_m \approx 0$ mV ($n = 1$). A small hysteresis effect was still observed in oxidative and reductive titrations although with the mediators used in Fig. 5, titrations in the oxidative and reductive direction gave essentially the same E_m, and no significant amount of low-potential (-100 mV) quenching component was detected in the PS II preparation. The result indicates that the primary electron acceptor Q_α of PS II_α in the Triton PS II preparation titrates with an E_m value of approx. 0 mV at pH 7.8.

The effect of DCMU concentration on the fluorescence yield of PS II is shown in Fig. 6. The variable portion of the fluorescence yield (F_v) is found to be significantly more sensitive to high DCMU concentrations than the nonvariable portion (F_0), which is only slightly affected. This effect is manifested as the decrease in the F_{max}/F_0 ratio. As shown in Fig. 6, the selective effect of DCMU quenching on the variable fluorescence yield F_v saturated at a ratio of DCMU/Chl of 1 : 4. A similar effect was detected with unfractionated chloroplasts, although in this case the effect of DCMU was much less pronounced (data not shown). The basis of this effect is not understood and is being investigated in more detail in our laboratory. The dependence of oxygen evolution to DCMU is shown in Fig. 7: A 90% inhibition is obtained at a DCMU concentration of approx. 10 μM which corresponds to a DCMU/Chl ratio of approx. 1. The residual O_2-evolution activity, observed at higher DCMU concentra-

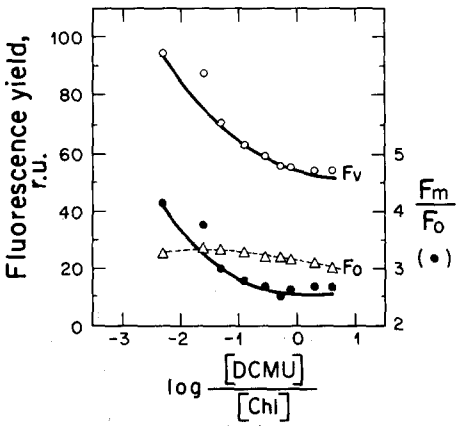

Fig. 6. Effect of DCMU on fluorescence yield of PS II. The assay conditions were essentially the same as in Fig. 3 except the amount of DCMU was varied as indicated. The presence of ethanol (less than 5%) was determined to have little effect on the fluorescence characteristics. The effects of increasing amounts of DCMU on the variable fluorescence, the initial fluorescence rise and the maximal fluorescence (F_v, F_0 and F_m, respectively) are shown. r.u., relative units.

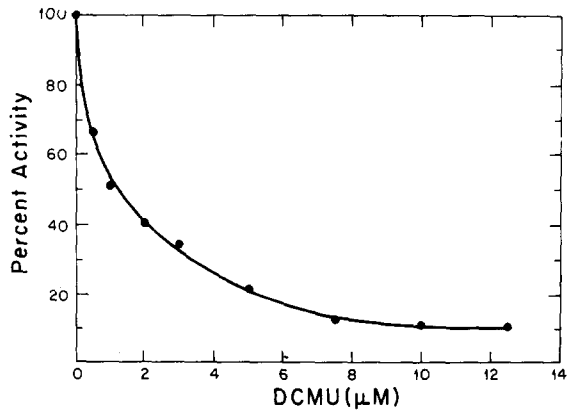

Fig. 7. Effect of DCMU on O_2 evolution in a PS II preparation. The assay medium contained 7.7 μg Chl/ml with 20 mM Mes (pH 6.0), 5 mM $MgCl_2$ and 15 mM NaCl. In addition, 3 mM potassium ferricyanide was added as acceptor prior to assay. The maximal activity (100%) was about 185 μmol oxygen atom evolved/mg Chl per h.

tions, suggests that potassium ferricyanide has a direct but limited accessibility to the primary electron acceptor Q of PS II in this preparation.

SDS-polyacrylamide gel electrophoresis analysis of polypeptide composition of the PS II preparation

The resolved PS II preparation was subjected to SDS-polyacrylamide gel electrophoresis analysis in order to characterize its peptide composition. The resulting gel was stained with Coomassie blue and the peptide pattern obtained shown in Fig. 8. Also shown in this figure is a scan recorded at 670 nm before staining with Coomassie blue in order to define the chlorophyll-containing bands in the preparation. There are at least 13 distinguishable peptides resolved by conventional SDS-polyacrylamide gel electrophoresis with relative molecular masses of 63, 55, 51, 48, 37, 33, 28, 27, 22, 15, 13 and 10 kDa. The 28 kDa peptide contained chlorophyll and, therefore, was subjected to further analysis for composition identification. We obtained an absorbance spectrum of a gel slice from this band which showed the typical PS II-Chl *a/b* LHCP spectrum (data not shown). Two other minor chlorophyll-containing bands (134 and 53 kDa) were observed under these conditions, but the small amounts of these bands precluded further analysis.

It was noted during the course of our studies that under the conventional SDS-polyacrylamide gel electrophoresis conditions, PS I was not completely solubilized as indicated by a large amount of CP Ia (data not shown). In addition, a significant amount of material did not migrate into the separation gel when a PS I preparation [14] was studied under identical conditions. These observations prompted us to suspect that some polypeptides, especially those of PS I, are inadequately solubilized under the conventional SDS-polyacrylamide gel electrophoresis conditions. After trying various treatments that might alleviate the problem (e.g., heating, sonication, etc.), we found that the inclusion of 2 M urea is the method of choice. The polypeptide pattern of the PS II preparation when analyzed by SDS-polyacrylamide gel electrophoresis in the presence of 2 M urea is shown in Fig. 9. Polypeptides with molecular masses of 47, 39, 31, 29, 27, 26, 23, 21, 17, 14, 13.6 and 13 kDa were observed. Under identical conditions, no CP Ia (molecular mass approx. 130 kDa) or aggregated materials were observed with either thylakoids or PS I preparation (data not shown). CP I migrates with a molecular mass of 60–63 kDa and no bound chlorophyll is observed under

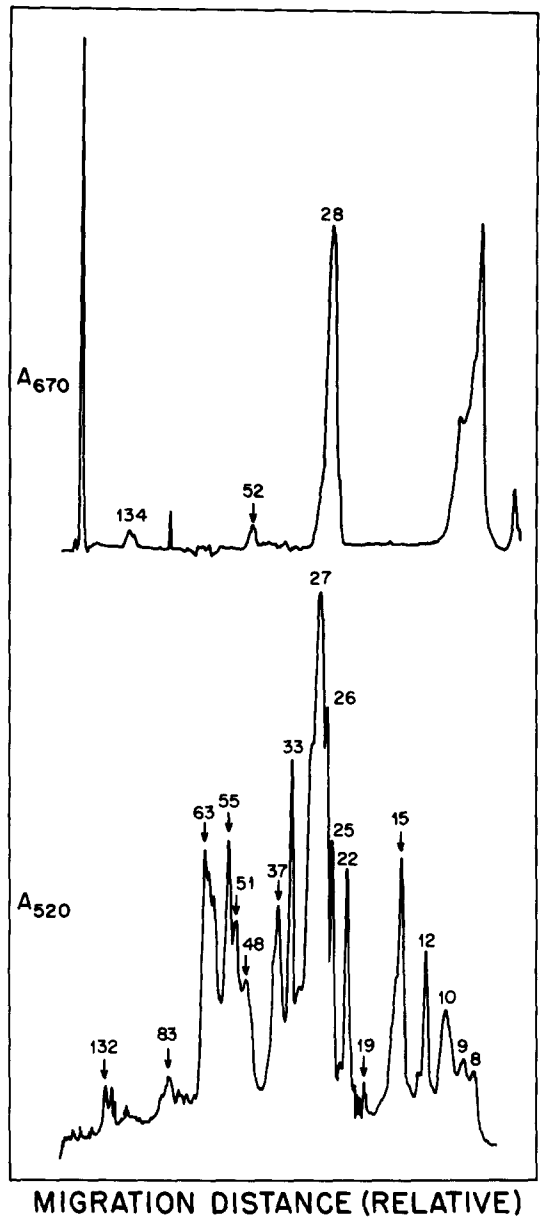

Fig. 8. SDS-polyacrylamide gel electrophoresis analysis of an O_2-evolving PS II preparation. The PS II preparation was treated as described in Ref. 20 for SDS-polyacrylamide gel electrophoresis analysis using a 10–15% linear acrylamide gel. The amount of chlorophyll in the sample was approx. 10 μg.

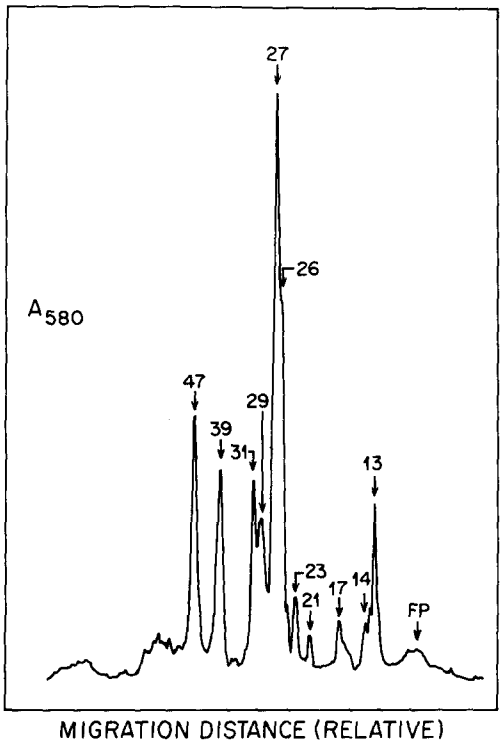

Fig. 9. SDS-polyacrylamide gel electrophoresis analysis of a PS II preparation in the presence of urea. The PS II preparation was analyzed as in Fig. 8 except 2 M urea was included in each step. No bound chlorophyll was observed after electrophoresis. FP, free pigment. 2 mM phenylmethylsulfonyl fluoride was also present during preparation of sample for electrophoresis.

these conditions. It is important to note the absence of any substantial amount of peptides in the 65–70 kDa range. In particular, no evidence for the PS I Chl *a*-protein, which should be present under the dissociating conditions used, could be obtained, and this result is in agreement with the absence of any significant amount of P-700 (PS I) in this PS II preparation.

Discussion

We have presented an analysis of the structural and functional components of a resolved Triton-PS II preparation from chloroplast thylakoids. Several functional features in this PS II preparation were identical to those of unfractionated thylakoids, suggesting that the PS II complex retained the native structural and functional organization of the unfractionated system. The water-splitting and electron-transport function of PS II has been retained in this preparation, including all electron-transport components from the water-splitting enzyme to the plastoquinone pool, which remained fully functional. An integral component of this preparation was also the cytochrome *b*-559 (cytochrome *b*-559/Q ratio equal to 2.0) while the PS I complex was missing (see Table I). The PS II preparation also contained the full complement of the chlorophyll antenna light-harvesting complex (Chl/Q = 230 ± 20).

The fractionation method employed in this work was essentially that of Berthold et al. [12] in which isolated thylakoids are suspended in a Mg^{2+}-containing medium and then incubated with Triton X-100. The presence of Mg^{2+} in the Triton incubation medium apparently helped to preserve the structural and functional integrity of PS II in the chloroplast partition region while it selectively solubilized the stroma-exposed thylakoids. This evaluation is supported by the observation that the PS II preparation retained the structural and functional integrity of PS II_α which is exclusively localized in the grana partition region [39]. The preparation also retained the PS II-LHCP, the component responsible for Signal II, and cytochrome *b*-559, all known to be associated with PS II. Should Triton have access to the PS II at the grana partition region, we would have expected the solubilization of the lipophilic plastoquinone pool by the nonionic detergent. In contrast, we measured the selective and quantitative removal of the cytochrome b_6-*f* complex (9% total final yield based on cytochrome *f* content) and the PS I complex (1% total final yield based on P-700 content), suggesting that these complexes are preferentially excluded from the membranes of the grana partition regions. The absence of PS I from the membranes of the grana partition region has been recently documented in several laboratories [39–43]. The localization of the cytochrome b_6-*f* complex in the thylakoid membrane is more controversial at present. Previous studies using inside-out vesicles derived from the grana partition regions and isolated stroma lamellae suggested equal distribution of the cytochrome complex in grana partition and stroma-exposed thylakoid membrane regions [42–45]. Our results indicate that the cyto-

chrome b_6-f complex is either not an integral component in the membrane of the grana partition regions or is selectively removed by the specific detergent treatment used. The former conclusion has been supported in a comparative study of the electron-transport components in mesophyll and bundle-sheath chloroplasts from maize [46]. Fully developed bundle sheath chloroplasts have lost exclusively, during the process of their differentiation, the structural entities of grana and the electron-transport components localized in them without losing any portion of the cytochrome b_6-f content [46]. Furthermore, it should be noted that a recent PS II vesicle preparation derived from the appressed region of the grana stacks by the polymer two-phase separation method [10] contained only negligible amounts of the cytochrome b_6-f complex while fully retaining most of the cytochrome b-559. In the latter case, almost half of the cytochrome b-559 was in its high-potential form while our Triton PS II preparation has a smaller high-potential component (approx. 20%). It has been suggested that the high-potential form of cytochrome b-559 is required for O_2 evolution [47], but our preparation retained high rates of O_2 evolution with only a small amount of the high-potential form of this cytochrome.

The PS II preparation also shows a large low-temperature light-induced EPR signal (Signal II) associated with the oxidizing side of PS II. Previous room-temperature measurements of Signal II reactions in the PS II preparation have been reported by Berthold et al. [12] while studies of low-temperature reactions have been described by Evans and co-workers [48,49]. Because of its high purity in relation to PS II components, the Triton preparation may prove useful in studying the functional role as well as the molecular identity of the component which yields the Signal II EPR spectrum.

The selective absence of PS II_β from our preparation further supports the notion of the localization of this photosystem in stroma-exposed thylakoids [39]. The kinetic analysis of PS II photoactivity revealed that the Triton-PS II preparation contained exclusively PS II_α. Several functional and organizational features of PS II_α were retained in the PS II preparation: the rate constant for light absorption and the nonlinear Q photoreduction kinetics. The former indicates that incubation with Triton has not altered the composition and size of the chlorophyll antenna associated with PS II_α while the latter points to the organization of PS II_α in aggregates of individual units in clusters, a structural and organizational feature of PS II_α known to occur in the grana membranes of unfractionated chloroplasts [35,50].

Potentiometric titrations of the fluorescence quencher in the past have identified two quenching components in unfractioned thylakoids, Q_H with $E_m \approx 0$ mV and Q_L with $E_m \approx -270$ mV [18,37,38]. Attempts have been made to associate the primary electron acceptor, Q_α, of PS II_α with the -270 mV component [37,51–53]. In the present work we have clearly shown that such a correlation is incorrect as we have measured an E_m value of approx. 0 mV for the primary electron acceptor Q of our PS II preparation, and we have shown the absence of Q_L (see Fig. 5). We attribute this marked difference in the measured E_m value of Q_α in chloroplasts and the Triton PS II preparation to greater accessibility of the primary electron acceptor in the PS II preparation to the redox mediators, since in unfractionated membranes PS II_α is localized in the hydrophobic appressed membrane regions to which polar redox mediators may have only limited accessibility. Accordingly, the true E_m value for the primary electron acceptor Q_α (PS II_α) is probably approx. 0 mV. Thus, the apparent E_m value of -270 mV of Q_L [37] may be explained as a titration of at least a portion of Q_α which has not adequately equilibrated with the redox mediators used for the E_m determination because these centers are located in the appressed membrane region.

Several groups have recently described thylakoid peptides specifically required for O_2 evolution. Spector and Winget [54] reported the requirement for a 65 kDa manganese-containing protein, while Nakatani and Barber [55] reported stimulation of O_2 evolution by a 58 kDa heme-containing peptide. Although peptides in this molecular mass range are present in the PS II preparation under conventional SDS-polyacrylamide gel electrophoresis conditions, some of these appear to be associated with contaminating coupling factor (CF_1), since treatments which remove CF_1 prior to Triton treatment decrease the amounts of the high molecular

mass components (unpublished observations). In any case, since these higher molecular mass peptides (greater than 50 kDa) were not observed when 2 M urea was present during SDS-polyacrylamide gel electrophoresis, we suggest that they might be aggregates of inadequately solubilized materials. Satoh [8] has shown that the reaction center pigment-protein complex of PS II consists of peptides with molecular masses of 43 and 27 kDa, while Laszlo and Gross [56] reported the presence of two PS II core complex peptides with molecular masses of 54 and 47 kDa. Both of these studies did not include urea in their SDS-polyacrylamide gel electrophoresis analysis. Under similar conditions, our preparation shows four peptides with molecular masses of 55, 48, 37 and 27 kDa which may be the components of the PS II reaction center core complex observed by these authors. However, only the polypeptides with molecular masses of 47, 39, 31, 29, 27 and 26 kDa were apparent after SDS-polyacrylamide gel electrophoresis with 2 M urea. One of the 25–27 kDa polypeptides appears to correspond to the LHCP of PS II. Of the remaining ones, the 47, 39 and 26 kDa polypeptides are suggested to constitute the reaction center complex of PS II as reported by others [8,56]. The 54 kDa polypeptide observed by Laszlo and Gross might be due to inadequate solubilization of the sample. The function of the remaining peptides, particularly those of low molecular mass, is uncertain but may be related to quinone binding and/or O_2 evolution. A role for a low molecular mass peptide in O_2 evolution has recently been reported by Akerlund et al. [57] who demonstrated a restoration of O_2 evolution by addition of a 23 kDa peptide after salt extraction. Recently, Toyoshima and Fukutaka [58] also reported that a protein with a molecular mass of 17 kDa is essential in O_2 evolution after cholate extraction of thylakoids.

In summary, because of the ease of preparation, high yield and apparent undamaged configuration of many of its PS II components, the oxygen-evolving PS II preparation should prove to be invaluable in further detailed analysis of the mechanism of primary charge separation and associated secondary electron-transfer events of PS II.

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