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Roles of three lumen-surface proteins in the formation of S_2 state and O_2 evolution in Photosystem II particles from spinach thylakoid membranes

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Reconstitution of O_2 evolution in $CaCl_2$-treated PS II at the level of the original PS II was achieved by simultaneous reinsertion of the three lumen-surface proteins, 17, 23 and 34 kDa proteins, and total thylakoid lipids in the presence of 25% glycerol and 15 mM sodium cholate. By means of this reconstitution method, the following was found, concerning the roles of the three proteins in the formation of the S_2 state and O_2 evolution of the water-splitting complex. (1) Complete removal of the three proteins from the PS II particles by 1 M $CaCl_2$ treatment caused a rapid release of Mn from the particles when incubated at 25°C, but not at 0°C. The rapid release of Mn at 25°C was prevented by the reinsertion of the 34 kDa protein but not by the other two proteins. (2) O_2 evolution was recovered in $CaCl_2$-treated PS II by reinserting the 34 kDa protein, when Cl^- was present at a level of 50 mM, but it declined when the Cl^- concentration was decreased. Upon further addition of the 23 kDa protein, O_2-evolving activity was enhanced to the original PS II level at concentrations of Cl^- higher than 10 mM. Below this concentration, besides the 23 and 34 kDa proteins, the 17 kDa protein was required for maximal O_2 evolution. (3) The effects of the three proteins and Cl^- on the recovery of the low-temperature multiline EPR signal in the $CaCl_2$-treated PS II were essentially the same as those on the recovery of the O_2 evolution, indicating that the three proteins and Cl^- contribute to the advancement of the water-splitting complex to the S_2 state.

Introduction

The central role of Mn in the water-splitting process has been widely acknowledged, but not only the chemical events involving Mn which lead to water splitting, but also the protein composition functionally and structurally connected with Mn is, in most respects, obscure. During the past 3 years, the constitution of the water-splitting complex in thylakoid membranes has been studied

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Abbreviations: PS II, Photosystem II; Chl, chlorophyll; P-680, photoreaction center of Photosystem II; FPLC, fast protein liquid chromatography; Mono Q, Pharmacia anion-exchange column for FPLC; Mono S, Pharmacia cation-exchange column for FPLC; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholinethanesulfonic acid; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; DPC, 1,5-diphenylcarbazide; DCIP, 2,6-dichlorophenolindophenol; PBQ, phenyl-*p*-benzoquinone.

mainly by means of the disintegration and reconstitution of O_2 evolution and it has been found that three water-soluble lumen-surface proteins, of 34 , 23 and 17 kDa, are involved in the water-splitting complex [1–18]. In early works, however, there were some discrepancies regarding the requirements of the three proteins in O_2 evolution [3,6,10,11,16–18]. The cause of discrepancies concerning the 17 and 23 kDa proteins has been clarified by the findings that the requirements for these proteins in O_2 evolution strongly depend on the Cl^- concentration in the assay medium for the O_2 evolution [13,15,18]. However, the discrepancies in the experimental findings regarding the role of the 34 kDa protein in the water-splitting complex have remained unsolved. Abramowicz and Dismukes [16] first reported the isolation of the 34 kDa protein through retention of Mn by using a redox buffer during protein extraction and later Yamamoto et al. [17] reported a successful isolation of the 34 kDa protein containing Mn by means of phase partitioning with 50% *n*-butanol. These results seem to suggest that the 34 kDa protein is a long-sought Mn-binding protein. However, Ono and Inoue [11] claimed that it is possible to dissociate the 34 kDa protein, together with the 17 and 23 kDa proteins, from the PS II-enriched thylakoid membranes (PS II particles) by washing with 1 M $CaCl_2$, leaving Mn unaffected and O_2 evolution partially restored in the $CaCl_2$-treated PS II particles, which results in the rebinding of the 34 kDa protein only. We also pointed out in a previous report that the recovery of O_2 evolution in the $CaCl_2$-treated particles was enhanced by further addition of the 17 and 23 kDa proteins in Ref. 18.

The experimental data on the requirements for the three proteins in photosynthetic water splitting have been collected from a number of groups, but there are serious discrepancies between the experimental results regarding the location of the effects of the 17, 23 and 34 kDa proteins in the charge-accumulation process of the water-splitting complex [19,20] Wensink et al. [19] compared DCIP reduction under flash illumination by the PS II particles before and after 2 M NaCl treatment and claimed that the 17 and 23 kDa proteins contribute to the S state transition higher than the $S_2 \rightarrow S_3$ transition. Investigating the S-state transition in the PS II particles treated with 1 M $CaCl_2$ by menas of thermoluminescence measurements, Ono and Inoue [20] provided evidence suggesting that the depletion of the three proteins leads to the inhibition of the $S_3 \rightarrow S_4$ transition, but not the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$ or $S_2 \rightarrow S_3$ transitions. We, however, reported previously that the formation of the light-induced multiline EPR signal attributed to the S_2 state is inhibited by removal of the 17 and 23 kDa proteins, when the Cl^- concentration is lower than 10 mM [21]. Casey and Sauer [22] presented evidence that formation of a pre-S_2 state observed by a 320 G wide EPR signal centered near $g = 4.1$ is inhibited by the removal of the 17 and 23 kDa proteins from the PS II particles.

In the present work, we have established a method to recover O_2 evolution in $CaCl_2$-treated PS II at the level of the original PS II and applied it to reveal the roles of the three proteins in the water-splitting complex, focusing on the following two points. (1) Relations among the three proteins and Cl^- for O_2 evolution and the formation of the EPR S_2 signal. (2) The effects of the three proteins on the stability of Mn in the native site of the water-splitting complex.

Besides reconstitution experiments, the effect of SO_4^{2-} on the formation of the S_2 state and O_2-evolving activity in the PS II particles have been examined.

Experimental procedure

Materials

O_2-evolving PS II particles were prepared from fresh spinach leaves by a method of Kuwabara and Murata [23] and finally suspended in a solution of 0.2 M sucrose, 20 mM NaCl and 20 mM Mops (pH 7.0) to be stored in liquid N_2.

The mixture of the 17, 23 and 34 kDa proteins was extracted from the PS II particles in 1 M $CaCl_2$ solution according to the method of Ono and Inoue [11]. After concentration in a dialysis bag (SPECTRAPOR membrane tubing, molecular mass cut-off 6–8 kDa) using poly(ethylene glycol) powder (molecular mass 20 ± 5 kDa), the solution containing the three proteins was dialyzed against buffer-solution A (0.2 M sucrose/20 mM Mops (pH 7.0)). This final solution was named as $CaCl_2$, from which 17, 23 and 34 kDa proteins were

isolated by using a Pharmacia FPLC system with columns of Mono Q for the 23 and 34 kDa proteins and Mono S for the 17 kDa protein as described previously [18]. Thylakoid total lipids were isolated from the broken thylakoids as shown in Ref. 15.

Depletion and reconstitution of PS II concerning the three proteins were carried out as follows. The PS II particles were washed with buffer-solution A and suspended in a solution of 1 M $CaCl_2$/0.3 M sorbitol/10 mM NaCl/40 mM Mes (pH 6.5) and at 1 mg/ml of Chl and stirred on ice for 30 min in the dark, followed by centrifugation at $30000 \times g$ for 15 min and at 4°C. The pellet was washed with buffer-solution A containing 20 mM sodium cholate at 0°C and at 2 mg Chl/ml to remove $CaCl_2$ from the pellet. The resulting pellet was named $CaCl_2$-treated PS II and used for the reconstitution experiments as follows. The pellet was resuspended at 4 mg Chl/ml in the buffer solution used for washing and homogenized at 0°C. To this suspension, the $CaCl_2$ extracts and thylakoid lipids were added at a ratio of proteins/lipids/Chl (3 : 6 : 2), otherwise mentioned together with glycerol and sodium cholate to give final concentrations of 25 vol% for glycerol, 15 mM for sodium cholate and 0.42 mg/ml for Chl. When the purified proteins were used for reconstitution instead of the $CaCl_2$ extracts, the ratio Chl/17 kDa/23 kDa/34 kDa was adjusted to 1.00/0.45/0.60/0.60 (w/w), otherwise mentioned. In the mixtures containing the $CaCl_2$-treated PS II, were various combinations of the three proteins and lipids, together with glycerol and sodium cholate, which we named the reconstitution mixture. After incubation at 4°C for 2 h under gentle shaking, the reconstitution mixture was diluted 50-times with the corresponding assay medium and centrifuged at $35000 \times g$ for 15 min. The resulting pellet, reconstituted PS II, was resuspended in each assay medium and submitted immediately to various measurements.

Complete removal of Cl^- from the PS II preparations was performed as follows. PS II particles were washed with buffer-solution A and immediately suspended in buffer-solution A plus 50 mM Na_2SO_4 at a Chl concentration of 30 μg/ml. After 1 min incubation in ice, the suspension was centrifuged at $35000 \times g$ for 15 min at 0°C. The resulting pellet was resuspended in the corresponding assay medium.

The determination of Mn content in the PS II and reconstituted PS II was carried out by means of atomic absorption spectrometry as shown in a previous report [18].

Measurements

The rate of O_2 evolution was measured with PS II and reconstituted PS II at 25°C and 0°C by use of a Teflon-covered electrode (Bionics Instrument) under continuous illumination with saturating light (0.17 W/cm^2) between 600 and 800 nm through a pair of glass filters (Toshiba IRA-25S and R-60) and a 10 cm water layer. PBQ was used as an electron acceptor.

The photoinduced electron transfer activities in the PS II and reconstituted PS II from DPC, as artificial electron donor, to DCIP, as artificial electron acceptor, and from H_2O to DCIP were determined by measuring the rate of DCIP photoreduction in the presence or absence of DPC, respectively, as described previously [15].

For EPR measurement of the original PS II and reconstituted PS II, the samples were washed in an appropriate buffer solution and suspended in buffer-solution A containing glycerol of spectroscopic grade at 4.0 mg Chl/ml with or without NaCl. For the Na_2SO_4-treated PS II, however, the final pellet was suspended in buffer-solution A containing glycerol, 50 mM Na_2SO_4 and 100 mM NaCl or in buffer-solution A cotnaining glycerol and 50 mM Na_2SO_4. In every preparation, the concentration of glycerol in the final suspension was 50 vol%. The sample solutions were placed in quartz tubes of 3 mm inner diameter and kept in the dark at 0°C for 60 min. The continuous illumination for the samples was performed as follows. After dark-adaptation, the sample tubes were equilibrated in the dark at 195 K in a glass Dewar's vessel containing solid CO_2 and methanol and illuminated with continuous light between 600 and 800 nm through a colored glass filter (Kenko SR 60) and a 10 cm water layer. Immediately after the illumination, the sample tubes were immersed in liquid N_2 and stored pending EPR measurement. Besides the continuous illumination experiments, laser-flash experiments were carried out by means of an Nd-YAG Laser (NEC SL120D) providing a

30 mJ and 3 ns laser flash at 534 nm. The samples were allowed to dark-adapt in EPR tubes at 0°C for 60 min and given one laser flash and immediately frozen in liquid N_2. Sample handling and measurement were completed within 24 h.

EPR spectra were recorded with a Varian E-109 system equipped with an Oxford EPR-900 continuous flow cryostat. The measurements were carried out at 8.0 ± 0.5 K and 4.6 ± 0.4 K. The microwave power was varied between 0.04 mW and 40 mW and the modulation amplitude was set at 5 G or 20 G. Other spectrometer conditions are indicated in the corresponding figure legends.

Results and Discussion

Perfect depletion and reconstitution of the 17, 23 and 34 kDa proteins for PS II particles

The composition of proteins obtained from PS II particles upon various treatment was examined by SDS-polyacrylamide gel electrophoresis. Although not depicted, comparison of the protein patterns of PS II before and after the $CaCl_2$ treatment indicated that treatment of PS II with 1 M $CaCl_2$ results in complete removal of the 17, 23 and 34 kDa proteins from PS II as reported in previous studies [15,21]. Fig. 1 shows the plots of the amounts of the three proteins involved in the different preparations of reconstituted PS II against the amounts of the corresponding protein added in the reconstitution mixture. Fig. 1a represents the data obtained in the reconstituted PS II prepared with each one of the purified proteins and the lipids, reconstituted PS II (17, ℓ), reconstituted PS II (23, ℓ), reconstituted PS II (34, ℓ). The amount of the 34 kDa protein in the reconstituted PS II (34, ℓ) saturated to the same level of the original PS II. However, that of the 23 kDa protein in the reconstituted PS II (23, ℓ) saturated to a level of 0.5 of the original PS II, while the 17 kDa protein could be rebound in the reconstituted PS II (17, ℓ) exceeding the original level. Fig. 1b shows the corresponding plots for reconstituted PS II prepared with the crude $CaCl_2$ extracts and the lipids. In this case, the saturation level of the 23 kDa protein in the reconstituted PS II was recovered to the level of the original PS II. The existence of the 34 kDa protein, rather than the 17 kDa protein, in the reconstitution medium was confirmed to be responsible for the perfect recovery in the binding of the 23 kDa protein to the reconstituted PS II. The removal of the thylakoid lipids from the reconstitution mixture did not give any significant effect on the protein patterns of the reconstituted PS II preparations.

Typical examples of the rate of O_2 evolution measured at 25°C with the PS II and reconstituted PS II prepared under various conditions are compared in Table I. The O_2-evolving activity of original PS II was in the range 300–450 μmol O_2/mg Chl per h, depending on delicate differences in the Triton X-100 treatment during the preparation of the PS II particles. After the treatment with 1 M $CaCl_2$, the PS II particles scarcely exhibited O_2 evolution in buffer-solution A containing 20 mM Cl^-. When reconstitution was carried out with the $CaCl_2$ extracts but without the lipids, O_2-evolving activity was partially restored in the particles, but did not exceed 50% of the original level, although, in this case, each of the 17, 23 and 34 kDa proteins was confirmed to be fully rebound to the reconstituted particles. The addition of the lipids to the reconstitution mixture containing $CaCl_2$ extracts significantly enhanced the O_2-evolving activity of the resulting reconstituted PS II and the recovery attained a maximum level at 3 mg lipids/mg Chl.

The rate of photoreduction of DCIP by PS II and reconstituted PS II in the presence and absence of DPC are also shown and compared with the O_2-evolving rates in Table I. With DPC as supplemental electron donor, the rate of DCIP photoreduction by the $CaCl_2$ treated PS II was 42% of the rate in original PS II. This rate increased to 85%, when reconstitution was carried out with the thylakoid lipids, reconstituted PS II(ℓ), but the rate of DCIP photoreduction without DPC and of O_2 evolution were not enhanced. These results suggest that the added thylakoid lipids contribute to restoring the electron-transfer activity from the DPC donation site to the DCIP-acceptance site, rather than reactivating the water-splitting complex.

It should be mentioned here that the O_2-evolution rate and the electron-transfer rate obtained in the reconstituted PS II prepared with the $CaCl_2$ extracts and lipids, reconstituted PS II ($CaCl_2$ extracts, ℓ) sometime exceeded the corresponding values in original PS II, as is shown in Table I.

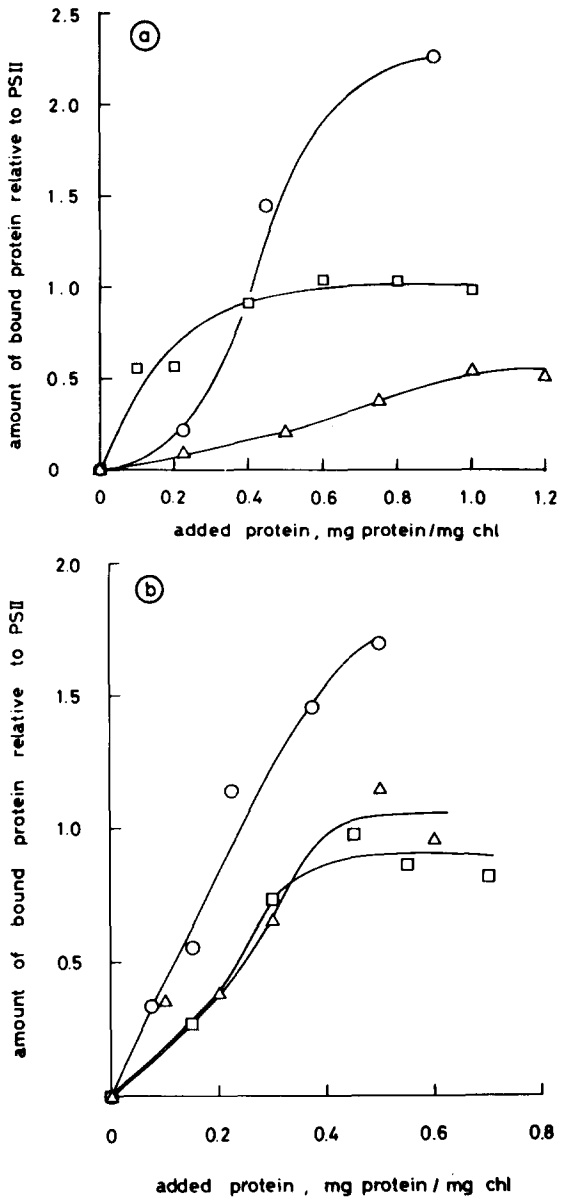

Fig. 1. Binding behaviour of the three proteins to different preparations of reconstituted PS II. Ordinate: the amount of each protein rebound to the reconstituted PS II relative to the amount of the corresponding protein in the original PS II, abscissa: the amount of the protein added to reconstituted PS II. (a) Data obtained from reconstituted PS II prepared with each one of the purified proteins: (○) 17 kDa protein; (△) 23 kDa protein; (□) 34 kDa protein. In this figure, the abscissa values represent the amount of each protein added in the reconstitution mixture. (b) Data obtained from reconstituted PS II prepared with the crude $CaCl_2$ extracts: (○) 17 kDa protein; (△) 23 kDa protein; (□) 34 kDa protein. In this figure, the abscissa values represent the amount of total proteins added in the reconstitution mixture.

This fact suggests that each of the 17, 23 and 34 kDa proteins and/or the lipid components functionally connected to PS II might be partially removed from the membranes during the preparation of the PS II particles. In fact, the activity of PS II, enriched with the three proteins and lipids by its incubating in a solution containing $CaCl_2$ extracts, lipids, 15 mM sodium cholate and 25 vol% glycerol, was in the range 420–580 μmol O_2/mg Chl per h, exceeding the value of original PS II particles.

Roles of the three lumen-surface proteins in O_2 evolution

The effects of the three proteins on the stability of Mn in the membranes were examined by determining the Mn content in the PS II, before and after $CaCl_2$ treatment, and in the different pre-

TABLE I

EFFECT OF THE THREE PROTEINS AND THE THYLAKOID LIPIDS ON ELECTRON TRANSFER RATES FROM DPC TO DCIP AND FROM H_2O TO DCIP, AND ON THE O_2-EVOLUTION RATES OF THE PS II, $CaCl_2$-TREATED PS II AND RECONSTITUTED PS II PREPARED UNDER DIFFERENT CONDITIONS.

All assays were carried out in buffer-solution A containing 20 mM NaCl: reconstituted PS II (ℓ), lipids/Chl ratio, 3.0/1.0; reconstituted PS II ($CaCl_2$ extracts), $CaCl_2$ extracts/Chl ratio, 1.5/1.0; reconstituted PS II ($CaCl_2$ extracts, ℓ), $CaCl_2$ extracts/lipids/Chl ratio, 1.5/3.0/1.0; Protein-enriched PS II, $CaCl_2$ extracts/lipids/Chl ratio 1.5/3.0/1.0. Measurements of DCIP reduction were carried out at a concentration of 50 μM DCIP and 25 μg Chl/ml with and without 250 μM DPC under illumination with 600–800 nm light at 0.15 mW/cm^2 and measurements of O_2 evolution were performed at 300 μM PBQ and 8.3 μg Chl/ml under illumination with saturating light of 600–800 nm (0.17 W/cm^2).

Sample	DCIP reduction (μequiv/mg Chl per h)		Oxygen evolution (μmol O_2/mg Chl per h)
	with DPC	without DPC	
Original PS II	38.5	32.2	312
$CaCl_2$-treated PS II	16.2	0.0	10
Reconstituted PS II (ℓ)	31.7	0.0	20
Reconstituted PS II ($CaCl_2$ extracts)	–	–	151
Reconstituted PS II ($CaCl_2$ extracts, ℓ)	44.1	39.0	350
Protein-enriched PS II	–	–	427

parations of reconstituted PS II. The PS II particles prepared by using Triton X-100 contained Mn in the range 3.8–4.0 atoms per 220 Chl, and just after the $CaCl_2$ treatment at 0°C, almost all of Mn still remained in the membranes, although the 17, 23 and 34 kDa proteins had been completely removed from the membranes. Furthermore, when the CaCl-treated PS II was resuspended in buffer-solution A containing 15 mM sodium cholate at 0.5 mg Chl/ml and incubated in the dark at 0°C, the release of Mn from the membranes was very slow, as shown in Fig. 2a. However, when the incubation was carried out at 25°C, an abrupt release of Mn from the membranes was observed. The corresponding results obtained with untreated PS II and the different preparations of reconstituted PS II are shown in Fig. 2b. In the membranes reconstituted with the three proteins and lipids, reconstituted PS II (17, 23, 34, ℓ), 3.8 Mn atoms per 220 Chl were retained, and warming them to 25°C did not cause the release of Mn. Similar results were obtained from membranes reconstituted with the 34 kDa protein and lipid, reconstituted PS II (34, l). Contrary to these results, reconstituted PS II lost 0.8 Mn atoms per 220 Chl during the reconstitution process and released Mn very rapidly when warmed to 25°C. Both the 17 and 23 kDa proteins were unable to substitute for the 34 kDa protein in the stabilization of Mn (data not shown). The addition of the three proteins to the reconstitution mixture after 1.5 h incubation at 25°C did not lead to rebinding of released Mn to the membranes.

As mentioned above, isolation of the 34 kDa protein with retention of Mn was reported by two different groups [16,17]. As regards the isoelectric aspect, the proteins isolated by the two groups are considered identical to the lumen-surface 34 kDa protein examined in the present work. From the results obtained in those studied, the 34 kDa protein was suggested to be a Mn-binding protein in the water-splitting complex. This idea, however, is inconsistent with the data obtained in the present work. As exhibited in Fig. 2a, the PS II particles completely depleted of the three proteins by $CaCl_2$ treatment can retain Mn at least for 4 h, as long as it is incubated at 0°C, although they had lost the O_2-evolving activity. Readdition of the three proteins to these depleted particles restored the O_2-evolving activity at almost the same level as in the original PS II, as will be shown later. Accordingly, after complete liberation of the three proteins from the membranes, Mn is considered able to remain in the native site of the water-splitting complex in a reversibly inactivated state. This inactivated Mn may be very labile compared with Mn in the

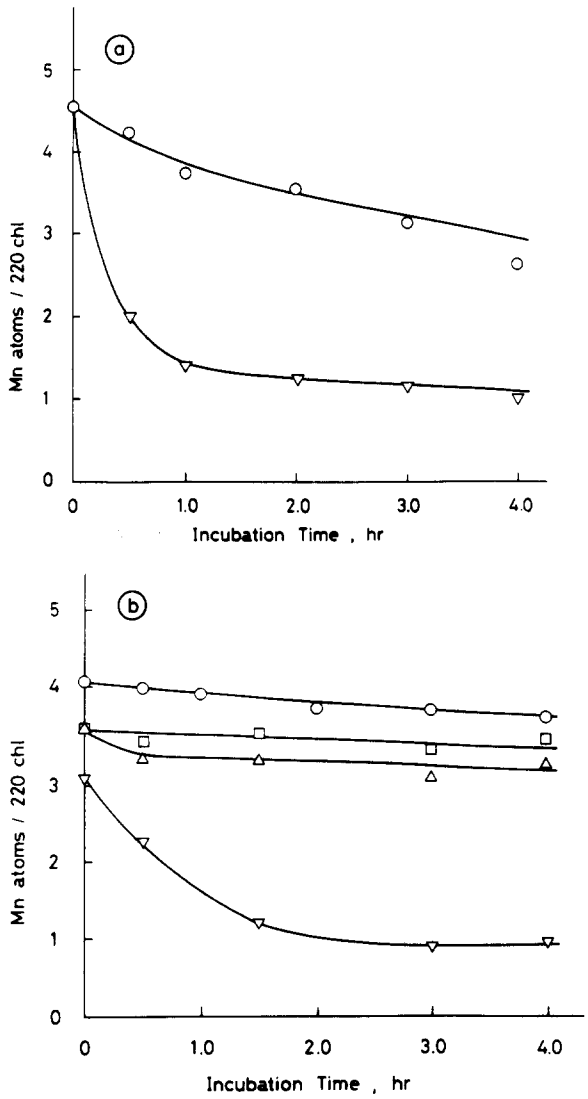

Fig. 2. Release of Mn from the PS II, $CaCl_2$-treated PS II and reconstituted PS II prepared under different conditions. (a) Release of Mn from the $CaCl_2$-treated PS II at temperatures 0°C (○) and 25°C (▽). (b) Release of Mn at 25°C from the PS II and from reconstituted PS II prepared under different conditions: (○) PS II; (□) reconstituted PS II (17, 23, 34, ℓ); (△) reconstituted PS II (34, ℓ); (▽) reconstituted PS II (ℓ).

native state, because contrary to the case of the original PS II, Mn in the PS II, lacking the 34 kDa protein, is rapidly released from the membranes by increasing the temperature. Consequently, the 34 kDa protein is not a Mn-binding protein, but it is inferred that it stabilizes a conformation of the water-splitting reaction center suitable for Mn to settle in the native sites.

The effects of the 34, 23, and 17 kDa proteins on the O_2 evolution were reexamined by comparing the restoration of the O_2-evolving activities of the different preparations of reconstituted PS II. The assay was done at 25 and 0°C, as a function of the Cl^- concentration. The results obtained at 25°C and shown in Fig. 3a clearly demonstrate the roles of the 17 and 23 kDa proteins in modifying Cl^- requirement on the activation of Mn in the water-splitting reaction center, in agreement with previous results [18]. The specific mechanism for the modification of Cl^- requirement on the activation of Mn in the water-splitting complex by the 23 and 17 kDa proteins is not yet clear. Certain amino-acid residues in these proteins themselves may act as Cl^- in activating Mn in the complex. Alternatively, the presence of these proteins in the membranes may increase the affinity for Cl^- of the water-splitting reaction center as suggested by Andersson [13] as the role of the 23 kDa protein. At the present stage, no possibility can be ruled out. The requirement of Cl^- observed in each reconstituted PS II was specific for Cl^- and not substituted by SO_4^{2-}. On the contrary, the addition of Na_2SO_4 to the assay medium at a level of 50 mM results in the significant reduction of O_2 evolution in each sample, including PS II, when Cl^- was not added in the assay medium. This inhibition of O_2 evolution by SO_4^{2-} was reversibly removed by the addition of Cl^-, suggesting that the inhibition is due to competition between SO_4^{2-} and Cl^- for the Cl^--binding site in the water-splitting complex. The present data shown in Table III for PS II qualitatively agree with the results reported by Itoh and Ueno [24].

Since Mn rapidly releases from the PS II particles depleted of the three proteins when kept at 25°C, the possibility that the loss of O_2-evolving activity of the $CaCl_2$-treated PS II at 25°C is due to the loss of Mn during the assay, rather than the depletion of the three proteins, still remains. This possibility may be checked by comparing the O_2-evolving activities of PS II and $CaCl_2$-treated PS II at 0°C. The data are shown in Fig. 3b, together

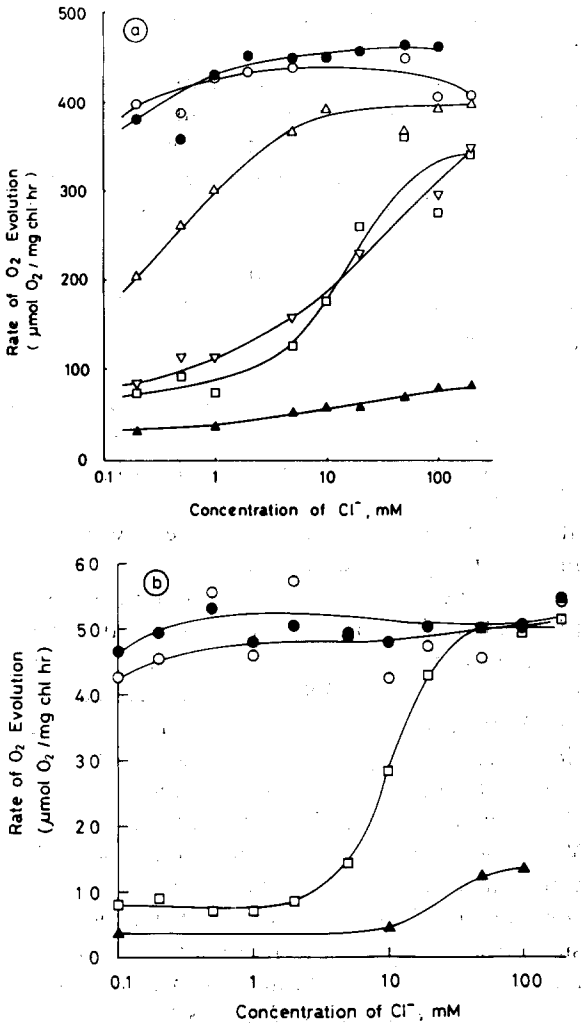

Fig. 3. Effect of Cl^- concentration on the O_2-evolving activity of PS II and reconstituted PS II, prepared under various conditions: (●) PS II particles (the particles were washed with buffer-solution A prior to the assay); (○) reconstituted PS II (17, 23, 34, ℓ), 17 kDa protein/23 kDa protein/34 kDa protein/lipids/Chl ratio, 0.45/0.60/0.60/3.0/1.0 (w/w); (△) reconstituted PS II (23, 34, ℓ), 23 kDa protein/34 kDa protein/lipids/Chl ratio, 0.60/0.60/3.0/1.0; (▽) reconstituted PS II (17, 34, ℓ), 17 kDa protein/34 kDa protein/lipids/Chl ratio, 0.45/0.60/3.0/1.0; (□) reconstituted PS II (34, ℓ), 34 kDa protein/lipids/Chl ratio, 0.60/3.0/1.0; (▲) reconstituted PS II (ℓ), lipids/Chl ratio, 3.0/1.0. The assays were carried out in buffer-solution A containing various amounts of NaCl. (a) Data obtained at 25°C. (b) Data obtained at 0°C.

with the results for reconstituted PS II (34, ℓ) and reconstituted PS II (17, 23, 34, ℓ). The results shown in the figure clearly indicate that the 34 kDa protein is required for not only the stabilization of Mn but also the functioning of the water-splitting complex. Although the absolute activity of each sample at 0°C is reasonably lower than the corresponding value at 25°C, the Cl^- concentration dependency of the activity obtained at 0°C is almost identical to that obtained at 25°C for all of the samples studied.

Effects of three proteins and Cl^- on the low-temperature EPR spectra of the water-splitting complex.

Fig. 4 shows the EPR spectra of PS II and the differently prepared reconstituted PS II observed at 8.0 ± 0.5 K and at a microwave power of 40 mW, in the presence of 20 mM Cl^-. As a reference, a spectrum for a non-illuminated PS II (dark-adapted) is included at the top of the figure together with noise level (spectrum A). Spectrum B was obtained with a PS II sample illuminated at 195 K for 5 min. As expected under these conditions [21,22,25–27], a large, well-resolved multiline signal attributed to the S_2 state of the water-splitting enzyme appeared between 2600 and 4000 G. When the temperature, Chl concentration and EPR tube diameter were fixed, the spectrum was reasonably reproducible both in line shape and intensity. Spectra C through G represent disappearance and recovery of the light-induced multiline signal upon various treatments of the PS II preparation. Corresponding results obtained with the samples to which no Cl^- was added are shown in Fig. 5. The light-induced multiline signal was observed in the PS II (spectrum A) and reconstituted PS II (17, 23, 34, ℓ) (spectrum E) without the addition of Cl^-, although the intensities were reduced by a factor of 1.7, respectively. While, for both reconstituted PS II (23, 34, ℓ) and reconstituted PS II (34, ℓ), removal of Cl^- from each sample solution caused the disappearance of the multiline signal which was observed in the presence of 20 mM Cl^- (spectra B and C, respectively).

The effect of the $CaCl_2$ treatment on the EPR spectrum of the PS II, measured after illumination by one short flash, is shown in Fig. 6. Comparing spectra A and B and A and C, one may observe that the periodic multiline signal designated by

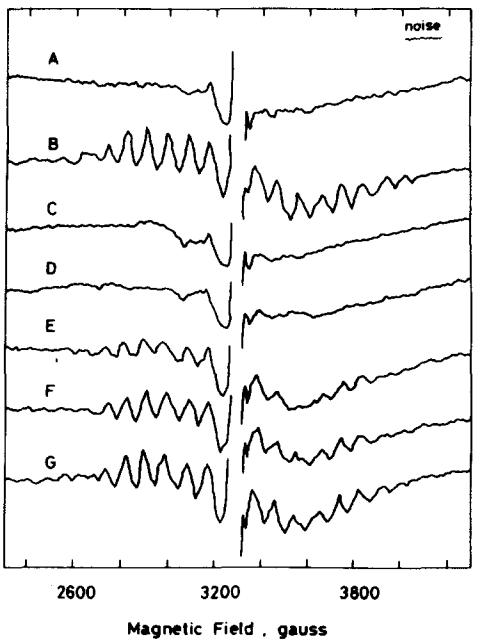

Fig. 4. EPR spectra of the PS II and differently prepared reconstituted PS II in the presence of 20 mM Cl^-. (A) Dark-adapted PS II at 273 K; (B) PS II illuminated; (C) reconstituted PS II (ℓ) illuminated; (D) reconstituted PS II (17, 23, ℓ) illuminated; (E) reconstituted PS II (34, ℓ) illuminated; (F) reconstituted PS II (23, 34, ℓ) illuminated; (G) reconstituted PS II (17, 23, 34, ℓ) illuminated. Illumination on each sample was carried out at 195 K for 5 min after dark-adaptation. EPR-measurement conditions were: temperature, 8.0 ± 0.5 K; microwave frequency, 9.240 GHz; power, 40 mW; modulation frequency, 100 kHz; modulation ampltiude, 20 G.

bars is developed in PS II by a single-flash illumination, but not in PS II treated with 1 M $CaCl_2$, although a considerably large irregular signal appeared. Thus, the removal of the three proteins seems to inhibit the $S_1 \rightarrow S_2$ transition of the water-splitting complex.

The effect of Na_2SO_4 treatment on the formation of the multiline EPR signal in the PS II particles was examined with microwave powers of 20 mW and 0.2 mW as is illustrated in Fig. 7. After treatment with 50 mM Na_2SO_4, the ability of PS II to develop a multiline signal was drastically reduced when Cl^- was not added in the sample solution (spectrum A), but it recovered by the addition of 100 mM NaCl at a level almost the same as that in the original PS II (spectrum B). In addition to this behavior, Mn(II) signal designated

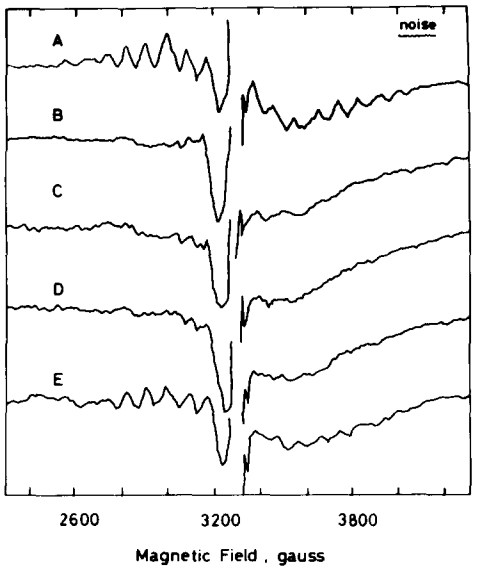

Fig. 5. EPR spectra of the PS II and differently prepared reconstituted PS II in the absence of Cl^-. (A) PS II illuminated; (B) reconstituted PS II (34, ℓ) illuminated; (C) reconstituted PS II (23, 34, ℓ) illuminated; (D) reconstituted PS II (17, 23, ℓ) illuminated; (E) reconstituted PS II (17, 23, 34, ℓ) illuminated. Illumination and EPR measurements were carried out as in Fig. 4.

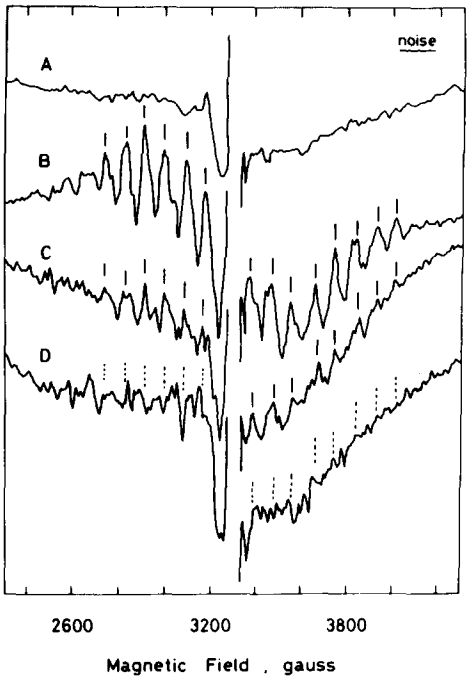

Fig. 6. Effect of the $CaCl_2$ treatment of PS II on the EPR spectrum obtained after one short flash illumination. (A) Dark-adapted PS II; (B) PS II illuminated for 5 min with continuous light at 195 K; (C) PS II illuminated with a single laser pulse at 273 K; (D) $CaCl_2$ treated PS II illuminated with a single laser pulse at 273 K. EPR-measurement conditions were: temperature, 4.6 ± 0.4 K; microwave frequency, 9.240 GHz; power, 20 mW; modulation frequency, 100 kHz; modulation amplitude 20 G.

by six arrows, appeared in the Na_2SO_4-treated PS II containing no Cl^- when the microwave power was set at 0.2 mW, but it was substituted by the multiline signal upon the addition of 100 mM NaCl.

In order to examine quantitatively the relationship between the intensity of the multiline signal and O_2-evolving activity, the signal intensity was estimated from the addition of the peak-to-peak heights of three well-defined low-field lines (2830, 2915 and 3005 G). In Table II, the results are summarized and compared with the O_2-evolving activity. As shown in the table, remarkable similarities in the protein requirements and Cl^--concentration dependency between the light-induced multiline signal intensity and the O_2-evolving activity were observed in the reconstituted PS II preparations. Apparent parallelism between the multiline signal and O_2-evolvin activity was noticeable also in the Na_2SO_4-treated PS II, as shown in Table III.

From the present findings shown in Figs. 4 and 5 and Tables II and III, we may propose that the 17, 23 and 34 kDa proteins participate in the formation of the S_2 state. This proposal is supported by the fact that the ability of PS II to develop the multiline signal upon illumination with a single flash after dark-adaptation is lost by the depletion of the three proteins (Fig. 6). Recently Franzen et al. [28] found that washing PS II particles with 1 M NaCl to remove the 17 and 23 kDa proteins induced an inhibition of the formation of the multiline signal, but Cl^- depletion of the PS II particles did not. From these findings, they suggested that the mechanism of the inhibition of O_2 evolution by NaCl washing does indeed differ from that by Cl^- depletion, and that the Cl^- depletion may inhibit the $S_2 \rightarrow S_3$ transition, although they pointed out the possibility of an alternative explanation. As mentioned before, it was not possible to exclude the presence of a small

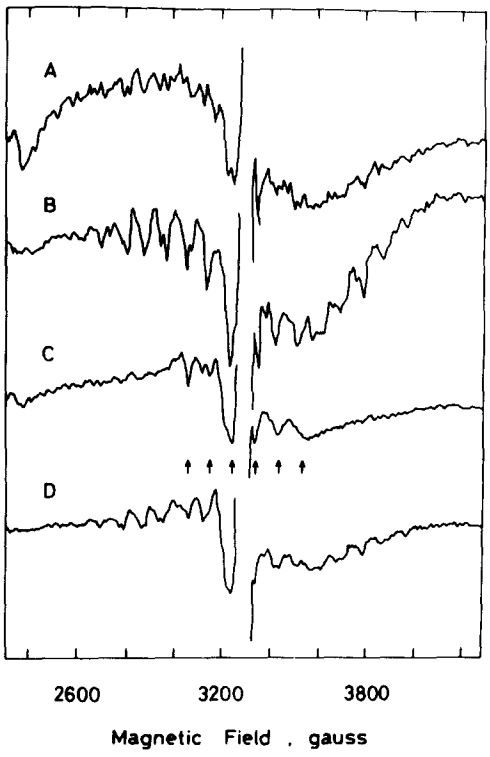

Fig. 7. EPR spectra of the Na_2SO_4-treated PS II with and without Cl^-. (A and C): Na_2SO_4-treated PS II without Cl^-; (B and D): Na_2SO_4-treated PS II with 100 mM Cl^-. Both samples were illuminated with continuous light for 5 min at 195 K. EPR-measurement conditions for A and B were: temperature, 8.0 ± 0.5 K; microwave frequency, 9.240 GHz; power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 20 G, and for C and D: temperature, 4.6 ± 0.4; microwave frequency, 9.240 GHz; power, 0.2 mW; modulation frequency, 100 kHz; modulation amplitude, 20 G.

TABLE III

COMPARISON OF THE EPR MULTILINE SIGNAL INTENSITY AND THE O_2-EVOLVING ACTIVITY IN PS II AND Na_2SO_4-TREATED PS II

EPR measurements were carried out at 8.0 ± 0.5 K and at a microwave power of 20 mW. The intensity of multiline signal for each sample was calculated relative to the value of the PS II in the presence of 50 mM Cl^-.

Sample	Oxygen evolution (μmol O_2/ mg Chl per h)	Signal intensity
PS II with 50 mM Cl^-	403	100
Na_2SO_4-treated PS II without Cl^-	52	trace
Na_2SO_4-treated PS II with 100 mM Cl^-	270	68

amount of tightly bound Cl^- in the PS II particles only by washing with a buffer solution containing no Cl^-, but an addition of SO_4^{2-} at a level of 50 mM to the resulting sample forced tightly bound Cl^- to release from the water-splitting complex. As shown in Fig. 7 and Table III, the complete depletion of Cl^- by the Na_2SO_4 treatment inhibits not only the O_2 evolution but also the formation of the S_2 state in the PS II and both inhibitions are removed by the readdition of Cl^-. From these results, we may conclude that O_2-evolving activity and the multiline EPR signal observed in the PS II

TABLE II

COMPARISON OF EPR MULTILINE SIGNAL INTENSITY AND THE O_2-EVOLVING ACTIVITY IN THE PS II AND DIFFERENT PREPARATIONS OF RECONSTITUTED PS II

EPR measurements were carried out at 8.0 ± 0.5 K and at a microwave power of 40 mW. The intensity of multiline signal for each sample was calculated relative to the value of the PS II in the presence of 20 mM Cl^-. n.d., not detectable.

Sample	20 mM NaCl		0.5 mM NaCl		No added NaCl	
	O_2 evolution (μmol O_2/ mg Chl per h)	signal intensity	O_2 evolution (μmol O_2/ mg Chl per h)	Signal intensity	O_2 evolution (μmol O_2/ mg Chl per h)	signal intensity
PS II	448	100	335	–	299	67
Reconstituted PS II (17, 23, 34, ℓ)	365	86	292	54	203	49
Reconstituted PS II (23, 34, ℓ)	315	68	104	18	52	n.d.
Reconstituted PS II (17, 34, ℓ)	111	35	75	n.d.	25	n.d.
Reconstituted PS II (17, 23, ℓ)	54	n.d.	32	n.d.	10	n.d.
Reconstituted PS II (34, ℓ)	125	41	74	n.d.	30	n.d.
Reconstituted PS II (23, ℓ)	55	n.d.	37	n.d.	10	n.d.
Reconstituted PS II (17, ℓ)	50	n.d.	35	n.d.	0	n.d.
Reconstituted PS II (ℓ)	57	n.d.	36	n.d.	0	n.d.

dispersed in the Cl^--free solution (see Tables II and III) and the multiline signal reported in Ref. 28 with Cl^--depleted PS II are due to the presence of strongly-bound Cl^- which may be closely associated with the reaction center Mn. Consequently, Cl^- is indeed required for the $S_1 \rightarrow S_2$ transition of the water-splitting complex as well as O_2 evolution. The present results concerning the roles of the three proteins in the formation of the S_2 state agree well with the results reported by Casey and Sauer [22] that the production of the 320 G wide EPR signal, which possibly originates from a pre-S_2 state, was inhibited by the depletion of the 17 and 23 kDa proteins, but they do not agree with the results of Wensink et al. [19], which suggested that the 17 and 23 kDa proteins contribute to an S state transition higher than the $S_2 \rightarrow S_3$ transition, nor with the results by Ono and Inoue [20] suggesting that the three proteins contribute to the $S_3 \rightarrow S_4$ transition. In a preliminary experiment concerning the effect of the 17 and 23 kDa proteins and Cl^- on the electron transfer from the water-splitting complex to the $P680^+$ produced upon a single flash illumination, we obtained evidence that the removal of these two proteins by washing with 1 M NaCl inhibits the $S_1 \rightarrow S_2$ transition of the water-splitting complex in the absence of Cl^-, and that this inhibition was partially removed by the addition of Cl^- to the sample solution. Details of the results will be published elsewhere. It is an advantage in the present experiments that the S_2 state was monitored directly by the presence of the multiline EPR signal, but further investigations of the intensity changes in the multiline EPR signal induced by a train of flashes are necessary for different preparations of reconstituted PS II in order to confirm the present results concerning the functional location of the proteins in the charge-accumulation steps.

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