Biochimica et Biophysica Acta 808 (1985) 171–179
Elsevier

BBA 41781

The roles of the extrinsic subunits in Photosystem II as revealed by EPR

Lars-Gunnar Franzén, Örjan Hansson and Lars-Erik Andréasson

Department of Biochemistry and Biophysics, Chalmers Institute of Technology and University of Göteborg, 41296 Göteborg (Sweden)

(Received January 17th, 1985)

Key words: Photosystem II; ESR; Electron transport

The effects of selective removal of extrinsic proteins on donor side electron transport in oxygen-evolving PS II particles were examined by monitoring the decay time of the EPR signal from the oxidized secondary donor, Z^+, and the amplitude of the multiline manganese EPR signal. Removal of the 16 and 24 kDa proteins by washing with 1 M NaCl inhibits oxygen evolution, but rapid electron transfer to Z^+ still occurs as evidenced by the near absence of Signal II_f. The absence of a multiline EPR signal shows that NaCl washing induces a modification of the oxygen-evolving complex which prevents the formation of the S_2 state. This modification is different from the one induced by chloride depletion of PS II particles, since in these a large multiline EPR signal is found. After removal of the 33 kDa protein with 1 M $MgCl_2$, Signal II_f is generated after a light flash. Readdition of the 33 kDa component to the depleted membranes accelerates the reduction of Z^+. Added calcium ions show a similar effect. These findings suggest that partial advancement through the oxygen-evolving cycle can occur in the absence of the 16 and 24 kDa proteins. The 33 kDa protein, on the other hand, may be necessary for such reactions to take place.

Introduction

During the past few years it has become increasingly evident that several extrinsic proteins, the 16, 24 and 33 kDa subunits, are involved in the process of transferring electrons from water on the donor side of Photosystem II. These conclusions have been arrived at through studies on preparations of Photosystem-II-enriched material with retained oxygen-evolving activity [1–3]. With these it has been possible to examine the inhibitory effects on oxygen evolution after the selective removal of the proteins by washing with NaCl (the 16 and 24 kDa proteins) [4,5] or $MgCl_2$, $CaCl_2$ or urea (all three proteins) [6,7]. Through such extraction experiments it is now clear that neither of the three proteins is directly responsible for the binding of photosynthetic manganese [4,6,8], although there is some evidence that the manganese binding site is prone to some labilization after removal of the 33 kDa protein [9].

It has recently been shown that the reconstitution of inhibited PS II particles by readdition of protein to extracted membranes is strongly dependent on the concentration of Cl^- in the medium [10]. This is consistent with earlier findings that Cl^- is required for a functional oxygen-evolving system [11–15]. Recent results also indicate that Cl^- stabilizes manganese after removal of the 33 kDa protein [9].

The importance of Cl^- in oxygen evolution is further augmented by the observation that Cl^- alone is capable of partially relieving the inhibition induced by the removal of the 16 and 24 kDa

Abbreviations: Chl, chlorophyll; Mes, 4-morpholineethanesulphonic acid; PPBQ, phenyl-*p*-benzoquinone; PS II, Photosystem II.

proteins [16,17]. Similarly, addition of Ca^{2+} to membranes depleted in these proteins leads to a significant recovery of oxygen-evolving activity and points to a role of this ion in donor side electron transport [17–19]. Consequently, it has been suggested that the 16 and 24 kDa components act to increase the affinity of the photosynthetic membranes for these ions [17,20,21].

Information about the consequences of protein removal on the mechanistic level has just recently emerged. After extraction of the 16 and 24 kDa proteins, Wensink et. al. [22] observed the transfer of two electrons to the acceptor side. Studies of the reduction of the secondary donor Z also indicate that some S-state transitions of the oxygen-evolving complex may occur in the absence of these proteins [23,24]. These effects are similar to those observed in Cl^--depleted chloroplasts where the inhibition of oxygen evolution is suggested to occur at the S_2–S_3 reaction step [25,26].

In the present work we have extended our previous studies [24] of the effects of selective removal of the above-mentioned proteins on the donor-side electron transport. From measurements on the EPR Signal II, due to the oxidized secondary donor Z^+, it appears that after removal of the 16 and 24 kDa proteins by NaCl washing, rapid electron transfer from the oxygen-evolving site may still occur, despite an almost complete inhibition of oxygen evolution. The efficiency of this transfer is considerably decreased when the 33 kDa protein is extracted. Readdition of the 33 kDa protein or the addition of Ca^{2+} to depleted PS II particles restores rapid electron donation to Z^+. The multiline EPR signal from state S_2 of the oxygen-evolving complex is eliminated after NaCl extraction, but not after inhibition of oxygen evolution induced by Cl^- depletion.

Some of the result herein were presented at the Third European Bioenergetics Conference (Hannover, 1984).

Materials and Methods

Photosystem II preparation. Oxygen-evolving Photosystem II particles were prepared as described in Ref. 27, with the exception that the stock solution of Triton X-100 (25% in 20 mM Mes (pH 6.3)/5 mM $MgCl_2$/15 mM NaCl) was added dropwise with stirring to the thylakoid suspension. The PS II particles were washed once with 20 mM Mes-NaOH (pH 6.3)/400 mM sucrose/5 mM $MgCl_2$/15 mM NaCl (25 mM Cl^- buffer) and suspended in 25 mM Cl^- buffer at 8–12 mg Chl/ml, and finally stored at 77 K until use. The oxygen-evolving activity was 500–600 μmol O_2/mg Chl per h and the manganese content 4.2 Mn/250 Chl.

Salt washings. The PS II particles were suspended in 1 M NaCl (or 1 M $MgCl_2$), 40 mM Mes-NaOH (pH 6.5) and 300 mM sorbitol at 0.5 mg Chl/ml, incubated on ice for 30 min and centrifuged at 40 000 × *g* for 20 min. The pellet from the NaCl treatment was washed twice with 25 mM Cl^- buffer or with 20 mM Mes-NaOH (pH 6.3), 400 mM sucrose, 5 mM $Mg(NO_3)_2$ and 1 mM NaCl (1 mM Cl^- buffer) and suspended at 3–7 mg Chl/ml in 25 mM Cl^- or 1 mM Cl^- buffer, respectively. The pellet from the $MgCl_2$ treatment was washed twice in 20 mM Mes-NaOH (pH 6.3)/400 mM sucrose/5 mM $MgCl_2$/190 mM NaCl (200 mM Cl^- buffer) and suspended at 3–7 mg Chl/ml in 200 mM Cl^- buffer. The high chloride concentration stabilizes manganese in the oxygen-evolving complex in the absence of the 33 kDa protein [9]. The salt washings and all handling of the salt-washed particles were performed in the dark to avoid photoinhibition.

Tris washing. The PS II particles were suspended in 0.8 M Tris (pH 8.4) at 1 mg Chl/ml, incubated in room light for 20 min at 0°C and centrifuged at 40 000 × *g* for 20 min. The pellet was suspended in 25 mM Cl^- buffer at 3–5 mg Chl/ml.

Chloride depletion. PS II particles (7 mg Chl) were incubated twice in 25 ml 20 mM Mes (pH 6.3)/5 mM $MgSO_4$ ('Cl^--free' buffer) for 15 min and pelleted by centrifugation at 40 000 × *g* for 20 min. For EPR experiments half of the material was suspended in the buffer diluted twice with glycerol, and the other half in the same glycerol-buffer mixture containing 25 mM NaCl. EDTA (1 mM) was present in both samples. All handling of the material was carried out in the dark.

Preparation of the 33 kDa protein. PS II particles were washed twice with 1 M NaCl as described above followed by washing with 1 M $MgCl_2$. The supernatant from the $MgCl_2$ washing

was concentrated by ultrafiltration with an Amicon PM10 Diaflo membrane and desalted on a Sephadex G-25 column equilibrated with 20 mM Mes-NaOH pH (6.3).

Activity measurements. Oxygen evolution was measured in saturating white light at 25°C with a Hansatech oxygen electrode in 20 mM Mes (pH 6.3), 400 mM sucrose and salts as indicated in the tables with 1 mM PPBQ as electron acceptor.

Laser flash experiments. PS II particles were suspended in a buffer containing 20 mM Mes (pH 6.3), 400 mM sucrose, 4 mM EDTA and salts as indicated in the figure legends and diluted twice with glycerol to 2 mg Chl/ml. The samples were allowed to dark-adapt in calibrated EPR tubes (inner diameter, 3 mm) at 0°C for at least 30 min before given a preflash from a dye (Rhodamin 6G) laser (Phase R DL-2100A2), and again allowed to dark-adapt for 5 min, now at 20°C. This procedure will place most of the material in the S_1 state [28]. The PS II particles were transferred to an ice bath, and PPBQ was added to a final concentration of 4 mM. Thereafter, the particles were given up to eight saturating laser flashes, spaced 2 s apart before beeing cooled to 200 K in an ethanol-dry ice bath, and finally frozen at 77 K. Before and after flashing the samples were handled in the dark.

Continuous illumination. Dark adapted PS II particles at 2–4 mg Chl/ml in the same medium as for the laser flash experiments were illuminated with white light in EPR tubes at 200 K in an ethanol-dry ice bath for 5 min before beeing frozen at 77 K. Alternatively, illumination during freezing was carried out as described in Ref. 29.

EPR measurements. Low temperature and room temperature EPR measurements were performed with the equipment described in Refs. 29 and 24, respectively. For the room temperature experiments, the PS II particles were suspended at 2–3 mg Chl/ml in a medium containing 20 mM Mes (pH 6.3), 400 mM sucrose, 1 mM EDTA and salts as indicated in the figure legends with ferro/ferricyanide (3 mM of each) as electron acceptor and 2 mM PPBQ as redox mediator. The samples were illuminated with saturating white flashes, half-width 3 μs and spaced 4 s apart, from a xenon flash lamp (Photochemical Research Associates Inc., model 610B). 25 flash-induced EPR transients, monitored at the Signal II low field peak at $g = 2.011$ with a sampling frequency of 250 Hz, were averaged and smoothed to an effective time resolution of 20 ms by taking the averages of five successive data points. The resulting transients were fitted with a non-linear least-squares method to a sum of a rising component (limited by the effective time resolution) and one or more decaying components.

Spin quantification. Signal II was quantified by comparison of its double integral with the one obtained from 0.1 M $VOSO_4$ in 1 M $HClO_4$ in the same sample cell. A proportionality between the Signal II amplitude at $g = 2.011$ and the double integral was found from measurements with several different Signal II concentrations. This was used in the quantification of Signal II_f.

$VO(H_2O)_5^{2+}$ has several advantages as an EPR concentration standard at room temperature: The optical extinction coefficient is known (16.5 $M^{-1} \cdot cm^{-1}$ at 765 nm [30]), it is easily prepared and it is stable. However, the many (approximately) Lorentzian hyperfine lines of ^{51}V (nuclear spin, $\frac{7}{2}$; $A = 0.011$ cm^{-1}) necessitate special precautions when integrating the EPR signal twice. In a separate investigation the relation $I = 6.80\ HW^2$ between the double integral of the whole spectrum divided by $g = 1.96$ (I) and the peak-to-peak height (H) and width (W) of the hyperfine line at the lowest field, was found to be satisfied, independent of temperature and vanadyl concentration. In subsequent spin concentration determinations, a measurement of H and W was thus sufficient.

Manganese analyses. Manganese was determined by EPR measurements at room-temperature on acidified samples using the method of additions.

SDS polyacrylamide gel electrophoresis. The samples were solubilized for 3 min at 80°C in 1% (w/v) SDS/5% (v/v) mercaptoethanol/1 mM EDTA/10% (w/v) sucrose/10 mM Tris-HCl (pH 8.0). Electrophoresis was performed in the buffer system of Laemmli [31], using 12–20% gradient gels (2.6% crosslinking). The gels were stained with Coomassie brilliant blue R-250.

Results

Effect of removal of proteins on manganese and Photosystem II electron transport

Oxygen-evolving PS II particles were treated with 1 M NaCl to remove specifically the 16 and 24 kDa extrinsic proteins or with 1 M $MgCl_2$ to liberate the 33 kDa protein in addition to the two lighter ones (Fig. 1). Both treatments resulted in a nearly complete inhibition of oxygen evolution (Table I). The inactivation was not caused by the release of functional manganese from the membrane, since the salt-treated membranes still contained about 4 Mn per functional unit (Table I).

The liberation of the three proteins did not inhibit the transfer of electrons from the added PS II donor diphenylcarbazide to 2,6-dichlorophenolindophenol (not shown). These results agree with the original findings of Ono and Inoue [6], and show that our PS II preparation is comparable to theirs. It was also possible to achieve partial reconstitution of oxygen evolution of the NaCl-washed particles by addition of Cl^- or Ca^{2+} (Table I) as observed previously [16–19]. A small reconstitution of oxygen evolution after addition of $CaCl_2$ to $MgCl_2$-washed particles was seen (Table I). This effect is possibly due to a small amount of residual 33 kDa protein and is analogous to $CaCl_2$ reconstitution in NaCl-washed PS II particles. We found that reconstitution was largely prevented if the depleted particles were exposed to strong light in the absence of Cl^- or Ca^{2+}. The presence of either of these ions suppressed light inactivation. Therefore, salt washing and handling of the depleted material was carried out in the dark.

TABLE I

EFFECTS OF SALT WASHINGS ON PS II PARTICLES

The control activity was 560 μmol O_2/mg Chl per h; n.d., not determined

	O_2 evolution (%)	Mn/250 Chl	Signal II_f (spins/250 Chl)
Control (25 mM Cl^-)	100	4.2	0.06
Tris-washed (25 mM Cl^-)	0	n.d.	0.71
NaCl-washed			
1 mM Cl^-	2	4.2	0.12
25 mM Cl^-	32	4.2	0.12
10 mM $CaCl_2$	37	4.2	0.10
$MgCl_2$-washed (200 mM Cl^-)	4	3.9	0.28
+33 kDa protein	8	3.9	0.05
+10 mM $CaCl_2$	10	3.9	0.24

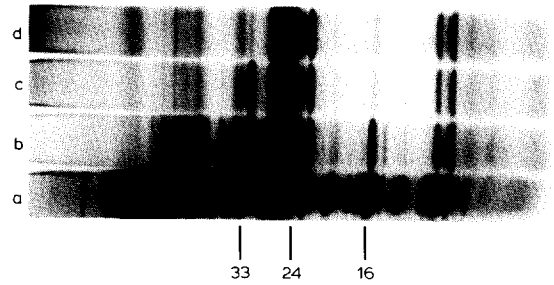

Fig. 1. Polypeptide analysis by electrophoresis in the presence of SDS. (a) Spinach thylakoids; (b) PS II particles; (c) NaCl-washed PS II particles; (d) $MgCl_2$-washed PS II particles.

Effect of salt washing on the formation of Signal II_f

With a functional oxygen-evolving system, the oxidized secondary PS II donor, Z^+, is rapidly reduced (usually within a few hundred μs) and observed in EPR as Signal II_{vf} [32]. Interruption of the flow of electrons from water by for example Tris-washing prevents rapid reduction of Z^+. Under these conditions, after a short light flash, Z^+ can be observed in EPR as Signal II_f with a life-time of a few hundred ms [33]. The results of the removal of the extrinsic proteins on the reduction of Z^+ are summarized in Figs. 2 and 3 and Table I. Due to limitations in time resolution by our instrumentation (more than 4 ms), Signal II_{vf} could not be studied directly, but the absence of a large Signal II_f after a flash given to the oxygen-evolving PS II particles is consistent with the rapid reduction of Z^+ (see Discussion). The amplitude of Signal II_f in oxygen-evolving material was generally less than 10% of that observed in the Tris-washed particles. Washing the PS II particles with 1 M NaCl to release the 16 and 24 kDa proteins did not result in a large increase in Signal II_f on the first or subsequent flashes (Fig. 2, Table I). However, if Tris was added to the NaCl-washed samples, the flash-induced EPR signal (not shown) was approximately equal in amplitude to that of the Tris-washed control. Even at 1 mM Cl^- where the oxygen-evolving activity was almost com-

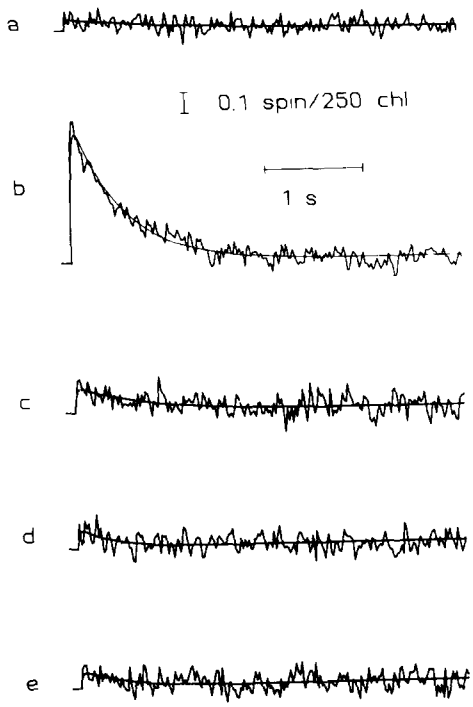

Fig. 2. Effect of NaCl washing on flash-induced EPR Signal II transients. (a) Oxygen-evolving PS II particles in 25 mM Cl^- buffer (control); (b) Tris-washed PS II particles in 25 mM Cl^- buffer; (c) NaCl-washed PS II particles in 1 mM Cl^- buffer; (d) NaCl-washed PS II particles in 25 mM Cl^- buffer; (e) as in (c), but after addition of 10 mM $CaCl_2$. All samples contained 1 mM EDTA, 3 mM of each of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ and 2 mM PPBQ. EPR conditions: magnetic field, 334.7 mT; microwave frequency, 9422 MHz; power, 2 mW; modulation amplitude, 0.25 mT; spectrometer time-constant, 3 ms; temperature, 20.0 ± 0.5°C.

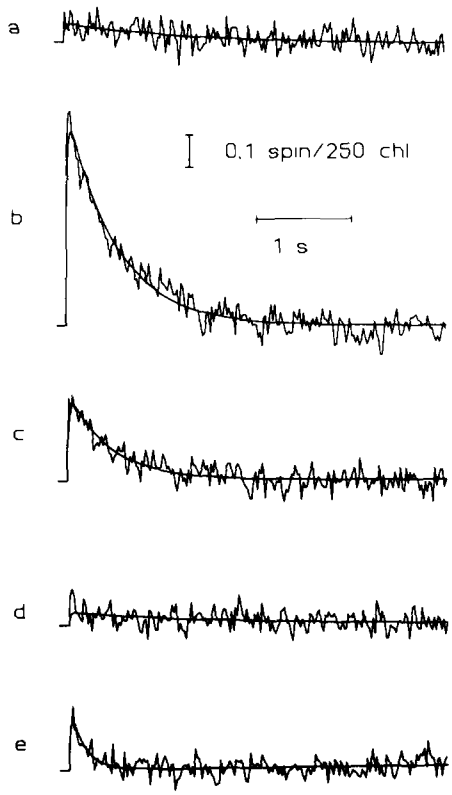

Fig. 3. Effect of $MgCl_2$ washing on flash-induced EPR Signal II transients. (a) Oxygen-evolving PS II particles in 25 mM Cl^- buffer (control); (b) Tris-washed PS II particles in 25 mM Cl^- buffer; (c) $MgCl_2$-washed PS II particles in 200 mM Cl^- buffer; (d) as in (c), but after addition of 0.2 mg purified 33 kDa protein/mg Chl; (e) as in (c), but after addition of 10 mM $CaCl_2$. Other conditions were as in Fig. 2.

pletely abolished, the amplitude of Signal II_f in NaCl-washed material was less than 20% of the Tris-induced signal and decreased slightly after addition of $CaCl_2$ (Table I).

PS II particles extracted with 1 M $MgCl_2$ to remove also the 33 kDa subunit showed a considerable amount of Signal II_f on the first and following flashes (Fig. 3, Table I). Readdition of the 33 kDa protein to the $MgCl_2$-washed particles led to only a small recovery of oxygen evolution, but to a decrease in Signal II_f to the control level (Fig. 3, Table I). This indicates that the reaction step leading to rapid reduction of Z^+ had been reactivated by the presence of the 33 kDa protein. After addition of $CaCl_2$ to PS II particles, the rate of reduction of Z^+ also increased (Fig. 3, Table I), but to a much smaller extent (half-time, 90 ms) than after reconstitution with the 33 kDa protein (halftime, less than 5 ms).

With oxygen-evolving PS II particles, with salt-washed particles or after Tris treatment, the decay of Signal II_f could be fitted to a single exponential decay with a half-time of about 200–500 ms. The traces presented in Figs. 2 and 3 have been smoothed to an effective time resolution of 20 ms per data point. There are no indications in the unsmoothed traces of Signal II transients with half-times in the 4–20 ms range (not shown).

Effect on the formation of the multiline EPR signal

Illumination of dark-adapted PS II particles by short light flashes leads to an oscillation of the population of the different S-states of the oxygen-evolving system [34]. One of these states, S_2, shows paramagnetic properties and exhibits a multiline EPR signal [29,35]. After freeze-trapping of PS II particles exposed to a series of light flashes at a temperature above 0°C, it can be shown that the S_2 EPR signal demonstrates a damped oscillatory behaviour [28,35], similar to that of the oxygen evolution, but with maxima on the first and fifth flash (Fig. 4a and c). After washing with 1 M NaCl to remove the 16 and 24 kDa proteins, the ability of the PS II particles to develop a multiline EPR signal as a response to one (Fig. 4b) or more light flashes was completely lost (not shown). Using continuous illumination at 200 K, which allows only the S_1–S_2 transition to take place [36], or during freezing to generate the multiline signal [29] yielded similar results, i.e., a large signal in the control which was almost absent in the NaCl-washed PS II particles (Fig. 5a and b). With the same techniques, no signal was seen after washing with 1 M $MgCl_2$ to remove the 33 kDa protein (not shown).

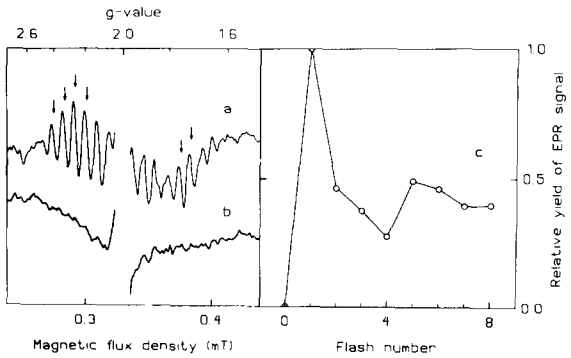

Fig. 4. Effect of NaCl washing on the flash-induced multiline EPR signal. Flash illumination and freezing of the samples were as described in Materials and Methods. (a) Single flash illumination of oxygen-evolving PS II particles in 25 mM Cl^- buffer, diluted twice with glycerol to 2.2 mg Chl/ml in the presence of 2 mM EDTA and 4 mM PPBQ; (b) as in (a), but NaCl-washed PS II particles in 1 mM Cl^- buffer (2.4 mg Chl/ml); (c) yield of multiline EPR signal after excitation of oxygen-evolving PS II particles with several flashes. EPR conditions: microwave frequency, 9.22 GHz; power, 20 mW; modulation amplitude, 2 mT; temperature, 8 K. Spectra from preflashed samples have been subtracted. The peak-to-peak heights of the six lines marked with arrows in (a) were summed for each sample and plotted in (c) after correction for tube calibration.

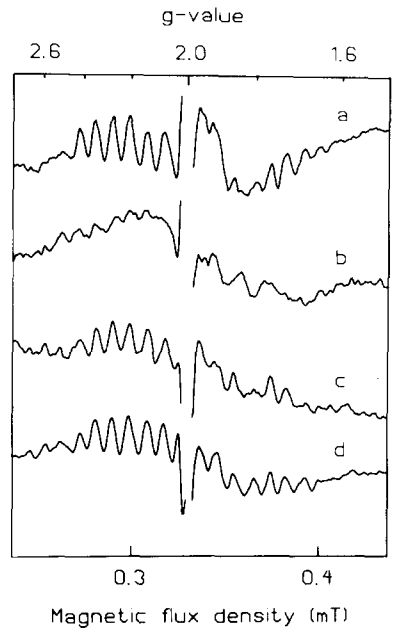

Fig. 5. Effect of NaCl washing and Cl^- depletion on the multiline EPR signal produced by continuous illumination at 200 K. Low temperature illumination were as described in Materials and Methods. (a) Oxygen-evolving PS II particles in 25 mM Cl^- buffer; (b) NaCl-washed PS II particles in 1 mM Cl^- buffer; (c) Cl^--depleted PS II particles in 'Cl^--free' buffer; (d) Cl^--depleted PS II particles replenished with 25 mM Cl^-. All samples were diluted twice with glycerol and contained 1–2 mM EDTA. EPR conditions were as in Fig. 4. The spectra presented are the differences between spectra obtained after and before illumination, normalized to the same chlorophyll concentration.

TABLE II

EFFECT OF CHLORIDE DEPLETION ON PS II PARTICLES

The control activity was 580 μmol O_2/mg Chl per h.

	O_2 evolution (%)	Multiline EPR signal, relative amplitude
Control (25 mM Cl^-)	100	1.0
Cl^--depleted (less than 25 μM Cl^-)	0	0.6
Cl^--replenished (25 mM Cl^-)	69	0.8

Comparison with Cl^--depleted material

Recent fluorescence induction experiments have indicated that the oxygen-evolving complex may be capable of limited electron transport after inhibition of oxygen evolution by chloride depletion [25,26], a treatment which may be analogous to the removal of the 16 and 24 kDa proteins. Accordingly, the ability of Cl^--depleted PS II particles to form the multiline EPR signal from state S_2 was examined by illumination at 200 K. Despite a complete inhibition of steady-state oxygen evolution (Table II), the capacity for the formation of the multiline EPR signal was nearly as good as in the oxygen-evolving control or in the particles where the oxygen evolution had been reconstituted by repletion of chloride (Fig. 5c and d). This behaviour deviates from that of the NaCl-washed particles described above.

Discussion

We have previously shown that inside-out thylakoid vesicles depleted in the 16 and 24 kDa proteins showed a Signal II_f, significantly smaller than that expected from the inhibition of oxygen evolution in addition to the lack of a multiline EPR signal [24], and suggested that the oxygen-evolving complex was capable of limited electron transfer to Z^+. Our results from PS II particles are fully consistent with this hypothesis. The low amplitude of Signal II_f after removal of the 16 and 24 kDa proteins indicates that the secondary donor Z is still rapidly reduced after a light flash. An alternative explanation for the absence of the EPR signal, namely the inability of P-680$^+$ to oxidize Z, is not likely. Although it has been shown recently that there is some effect on the rate of electron transfer between Z and P-680 as a result of the removal of the 16 and 24 kDa proteins [37], there is no complete block [38]. Also, when the liberation of the two subunits by NaCl is followed by Tris treatment, which extracts functional manganese and the 33 kDa protein, a normal Signal II_f is seen. This clearly shows that the Z–P-680 pathway remains operational.

A few other possible causes for the low amplitude of Signal II_f may also be excluded, such as the rapid reaction (below the time resolution of our instrument, 4 ms) with extraneous manganese (II) [39], since this should be prevented by the presence of EDTA. Such an effect of EDTA was observed by Ghanotakis et al. [23]. Cytochrome *b*-559 is also unlikely to act as reductant of Z^+, since the cytochrome is present in its oxidized form at the reduction potential of the medium (about 400 mV). Since no rapid phase in Signal II reduction is seen in Tris-washed samples where the donation from the oxygen-evolving complex is completely eliminated, other external reductants, such as $Fe(CN)_6^{4-}$, in the redox buffer may probably also be excluded.

Ghanotakis et al. observed a gradual increase in Signal II_f amplitude after NaCl washing as a response to flashes [23], in contrast to our results. One explanation for the discrepancy may be that their salt-washing procedure introduced additional modifications which prevented complete reduction of the donor to Z^+ between the flashes. For example, exposure to light of the salt-washed material eventually induces irreversible changes on the donor side which prevent reconstitution of oxygen evolution by Cl^- or Ca^{2+}. Dekker et al. [40] also observed that prolonged illumination of salt-washed PS II particles resulted in an inhibition of electron transfer from the oxygen-evolving complex. Similar observations were made by Nakatani [17]. In our EPR measurements only 25 saturating flashes were used, which is far below the threshold for photoinhibition [40]. Even with 50 flashes there were no signs of deleterious effects on the electron donation from the oxygen-evolving site, as evidenced by the constant, low amplitude of Signal II_f. However, our oxygen evolution data are consistent with measurements avoiding prolonged illumination. In the cases where flash-dependent oxygen yields actually have been measured with salt-washed material, severe effects were seen already after the first few flashes [22,41].

Thus, the most likely cause for the absence of Signal II_f in salt-washed material is a partially functional oxygen-evolving complex where cyclic turnover and oxygen evolution is prevented. This hypothesis raises the question of which step in the oxygen evolution cycle is being inhibited by the removal of the 16 and 24 kDa proteins. If it is assumed that the dark-adapted samples reside mainly in the S_1 state, the rapid reduction of Z^+ after a light flash clearly shows that the S_1–S_2 step

must be functional. However, we are unable to trap the S_2 state in NaCl-washed particles after flash illumination at 0°C or after freezing during illumination [24]. Also, the multiline signal is considerably decreased after low temperature (200 K) illumination (Fig. 5b) [42] which normally permits only the S_1–S_2 transition of the oxygen-evolving complex to occur and furthermore stabilizes the S_2 state towards relaxation back to S_1 [36]. Possibly, the stability of the S_2 state has decreased to such an extent as a result of salt washing that it relaxes back to the S_1 state between the flashes (i.e., in less than 4 s), and is furthermore unable to accumulate even at low temperatures. Such a relaxation may be the result of a recombination of the $S_2Q_A^-$ state [43]. Alternatively, salt washing may induce changes in the oxygen-evolving complex which affect the properties of the EPR signal from S_2 to such a degree that it is no longer recognizable (an alternative suggested to us by A.W. Rutherford, Centre d'Etudes Nucleaires de Saclay Gif-sur-Yvette, France), and instead influence the efficiency of the higher S-state transitions as suggested in Ref. 22. In a mixed valence Mn pair model the loss of the multiline signal could be achieved by a decrease in the exchange coupling between the two ions to a level comparable with the zero-field splitting and/or the Zeeman interaction of the individual ions [44].

There is yet another explanation of our observations. The NaCl washing may disconnect the oxygen-evolving complex completely but a rereducible intermediary carrier between the oxygen-evolving complex and Z is responsible for the rapid reduction of Z^+. Studies of Z^+ reduction in oxygen-evolving PS II particles [45] have indicated the presence of such a component. This may be related to the recently discovered EPR signal at $g = 4.1$ [28,46]. The possibility of such an intermediary, however, must be left open, since other recent results seem to preclude its existence [26]. Studies on Signal II_f generation after excitation with several, closely spaced, flashes could give kinetic information about the species responsible for the reduction of Z^+ and facilitate its identification.

It is interesting to compare the results of NaCl washing with that of Cl^- depletion, which also appears to induce modifications which allow only limited electron transfer from the oxygen-evolving complex [25,26]. Studies of Cl^--depleted PS II particles show that the reduction of Z^+ is inhibited to a significant extent [47] in contrast to our observation of NaCl-washed particles. The presence of a multiline EPR signal in Cl^--depleted PS II particles shows that the mechanism of inhibition is indeed different from that after NaCl washing and is consistent with an inhibition of the S_2–S_3 transition as suggested by fluorescence induction experiments [25,26]. As we deliberately avoided exposure of our samples to light to prevent photoinactivation, we cannot completely exclude the presence of a small amount of tightly bound Cl^- in the EPR sample in the case complete Cl^- depletion is light-dependent. Thus, an alternative explanation of the multiline signal would be that there actually exists such a strong-binding Cl^- site, maybe closely associated with the manganese. This possibility and the differences between NaCl washing and Cl^- depletion will be subjected to further investigations.

Extraction of the 33 kDa protein severely inhibits the electron transfer to Z^+ from its donor. This is clearly demonstrated by the development of Signal II_f after washing with 1 M $MgCl_2$. The situation differs from that after a Tris wash when the photosynthetic manganese is removed together with the 16, 24 and 33 kDa proteins. The removal of the 33 kDa protein may completely disconnect the oxygen-evolving complex, or a possible intermediate carrier, from Z^+. Readdition of the 33 kDa protein to the depleted membranes leads to an enhancement of the rate of the inhibited reaction step as shown by the low amplitude of Signal II_f. The observation that Ca^{2+} also accelerates the reduction of Z^+ may indicate an increased accessibility for an exogeneous donor [48], but it may also suggest that one role of the 33 kDa protein may be to provide positive charges needed for a functional electron transfer on the donor side of PS II.

Acknowledgements

We are indepted to professor Tore Vänngård for his helpful criticism. We also thank Mr. Mats Stading and Mr. Peter Brzezinski for help with the laser installation. This work was supported by the

Victor and Erna Hasselblad Foundation (purchase of the laser) and the Swedish Natural Science Research Council.

References

1 Åkerlund, H.-E., Andersson, B. and Albertsson, P.-Å. (1976) Biochim. Biophys. Acta 449, 525–535
2 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234
3 Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539
4 Åkerlund, H.-E., Jansson, C and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10
5 Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93
6 Ono, T.-A. and Inoue, Y. (1983) FEBS Lett. 164, 255–260
7 Miyao, M. and Murata, N. (1984) Biochim. Biophys. Acta 765, 253–257
8 Franzén, L.-G. and Andréasson, L.-E. (1984) Biochim. Biophys. Acta 765, 166–170
9 Miyao, M. and Murata, N. (1984) FEBS Lett. 170, 350–354
10 Andersson, B., Critchley, C., Ryrie, I.J., Jansson, C., Larsson, C. and Anderson, J.M. (1984) FEBS Lett. 168, 113–117
11 Warburg, O. and Luttgens, W. (1944) Naturwissenschaften 32, 301
12 Hind, G., Nakatani, H.Y. and Izawa. S. (1969) Biochim. Biophys. Acta 172, 277–289
13 Kelly, P.M. and Izawa, S. (1978) Biochim. Biophys. Acta 502, 198–210
14 Theg, S.M. and Hohmann, P.H. (1982) Biochim. Biophys. Acta 679, 221–234
15 Critchley, C. (1983) Biochim. Biophys. Acta 724, 1–5
16 Imaoka, A., Yanagi, M., Akabori, K. Toyoshima, Y. (1984) FEBS Lett. 176, 341–345
17 Nakatani, H.Y. (1984) Biochem. Biophys. Res. Commun. 120, 299–304
18 Miyao, M. and Murata, N. (1984) FEBS Lett. 168, 118–120
19 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 167, 127–130
20 Akabori, K., Imaoka, A. and Toyoshima, Y. (1984) FEBS Lett. 173, 36–40
21 Ghanotakis, D.F., Topper, J.N., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 170, 169–173
22 Wensink, J., Dekker, J.P. and Van Gorkom, H.J. (1984) Biochim. Biophys. Acta 765, 147–155
23 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) Biochim. Biophys. Acta 765, 388–398
24 Jansson, C., Hansson, Ö., Åkerlund, H.-E. and Andréasson, L.-E. (1984) Biochem. Biophys. Res. Commun. 124, 269–276
25 Theg, S.M., Jursinic, P.A. and Homann, P.H. (1984) Biochim. Biophys. Acta 766, 636–646
26 Itoh, S., Yerkes, C.T., Koike, H., Robinson, H.H. and Crofts, A.R. (1984) Biochim. Biophys. Acta 766, 612–622
27 Ford, R.C. and Evans, M.C.W. (1983) FEBS Lett. 160, 159–164
28 Zimmermann, J.L. and Rutherford, A.W. (1984) Biochim. Biophys. Acta 767, 160–167
29 Hansson, Ö. and Andréasson, L.-E. (1982) Biochim. Biophys. Acta 679, 261–268
30 Jørgensen, C.K. (1957) Acta Chem. Scand. 11, 73–85
31 Laemmli, U.K. (1970) Nature 227, 680–685
32 Blankenship, R.E., Babcock, G.T., Warden, J.T. and Sauer, K. (1975) FEBS Lett. 51, 287–293
33 Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 315–328
34 Kok, B., Forbush, B. and MgGloin, M. (1970) Photochem. Photobiol. 11, 457–475
35 Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274–278
36 Brudvig, G.W., Casey, J.L. and Sauer, K. (1983) Biochim. Biophys. Acta 723, 366–371
37 Åkerlund, H.-E., Brettel, K. and Witt, H.T. (1984) Biochim. Biophys. Acta 765, 7–11
38 Renger, G. and Völker, M. (1982) FEBS Lett. 149, 203–207
39 Babcock, G.T. and Sauer, K (1975) Biochim. Biophys. Acta 396, 48–62
40 Dekker, J.P., Ghanothakis, D.F., Plijter, J.J., Van Gorkom, H.J. and Babcock, G.T. (1984) Biochim. Biophys. Acta 767, 515–523
41 Åkerlund, H.-E. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 391–394. Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
42 Blough, N.V. and Sauer, K. (1984) Biochim. Biophys. Acta 767, 377–381
43 Rutherford, A.W. and Inoue, Y. (1983) FEBS Lett. 165, 163–170
44 Andréasson, L.-E., Hansson, Ö. and Vänngård, T. (1983) Chem. Scr. 21, 71–74
45 Boska, M. and Sauer, K. (1984) Biochim. Biophys. Acta 765, 84–87
46 Casey, J.L. and Sauer, K. (1984) Biohim. Biophys. Acta 767, 21–28
47 Babcock, G.T., Buttner, W.J., Ghanotakis, D.F., O'Malley, P.J., Yerkes, C.T. and Yocum, C.F. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 243–252, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
48 Yerkes, C.T. and Babcock, G.T. (1981) Biochim. Biophys. Acta 634, 19–29