Effect of the 17- and 23-Kilodalton Polypeptides, Calcium, and Chloride on Electron Transfer in Photosystem II†

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ABSTRACT: Electron paramagnetic resonance (EPR) measurements were performed on photosystem II (PSII) membranes that were treated with 2 M NaCl to release the 17- and 23-kilodalton (kDa) polypeptides. By using 75 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea to limit the photosystem II samples to one stable charge separation in the temperature range of 77–273 K, we have quantitated the EPR signals of the several electron donors and acceptors of photosystem II. It was found that removal of the 17- and 23-kDa polypeptides caused low potential cytochrome b_{559} to become fully oxidized during the course of dark adaptation. Following illumination at 77–130 K, one chlorophyll molecule per reaction center was oxidized. Between 130 and 200 K, both a chlorophyll molecule and the S_1 state were photooxidized and, together, accounted for one oxidation per reaction center. Above 200 K, the chlorophyll radical was unstable. Oxidation of the S_1 state gave rise to the S_2-state multiline EPR signal, which arises from the Mn site of the O_2-evolving center. The yield of the S_2-state multiline EPR signal in NaCl-washed PSII membranes was as high as 93% of the control, untreated PSII membranes, provided that both Ca^{2+} and Cl^- were bound. Furthermore, the ^{55}Mn nuclear hyperfine structure of the S_2-state multiline EPR signal was unaltered upon depletion of the 17- and 23-kDa polypeptides. In NaCl-washed PSII samples where Ca^{2+} and/or Cl^- were removed, however, the intensity of the S_2-state multiline EPR signal decreased in parallel with the fraction of PSII lacking bound Ca^{2+} and Cl^-. Reconstitution of Ca^{2+} was accomplished in a medium containing 100 mM NaCl, and the yield of the S_2-state multiline EPR signal after Ca^{2+} reconstitution was comparable to that observed in NaCl-washed PSII membranes before removal of Ca^{2+}. We conclude that Ca^{2+} and Cl^-, and not the 17- and 23-kDa polypeptides, are the main factors governing the ability to observe the S_2-state multiline EPR signal. Furthermore, removal of the 17- and 23-kDa polypeptides from photosystem II does not significantly perturb the environment of the Mn site of the O_2-evolving center.

Over the years, a considerable amount of evidence has been accumulated on the factors that lead to the proper functioning of the O_2-evolving center (OEC)[1] of photosystem II (PSII) of higher plants, but a cohesive picture of the water oxidation process has not yet emerged. It is known that Mn is essential for O_2 evolution to occur [for a review, see Amesz (1983)]. Also, optimal O_2 evolution activity is dependent on the presence of the 17- and 23-kDa polypeptides, which can be removed from inside-out thylakoids and PSII membranes by treatment with NaCl (Åkerlund et al., 1982; Miyao & Murata, 1983; Ghanotakis et al., 1984a) without loss of functional Mn (Ghanotakis et al., 1984a). Furthermore, in the absence of Ca^{2+} and Cl^-, PSII membranes that are depleted of the 17- and 23-kDa polypeptides show very low rates of O_2 evolution. Ghanotakis et al. (1984b) have shown that approximately 80% of the O_2 evolution activity of PSII membranes, which were depleted of the 17- and 23-kDa polypeptides, could be reconstituted by addition of 15 mM $CaCl_2$. A specific Cl^- effect has also been reported by Imaoka et al. (1984) and Miyao and Murata (1985) in PSII membranes that were depleted of the 17- and 23-kDa polypeptides.

The role of these polypeptides, Ca^{2+}, and Cl^- in the mechanism of O_2 evolution is a matter of considerable interest. In the Kok model for O_2 evolution, the photooxidation of H_2O to O_2 by PSII occurs via the formation of five intermediate oxidation states called S_i (i = 0–4) states (Kok et al., 1970), thus designated in order to reflect the number of oxidizing equivalents stored in the OEC by successive charge separations in the PSII reaction center. The S_4 state releases O_2 and is rapidly converted into the S_0 state. The S_2 and S_3 states are unstable and deactivate to the S_1 state within minutes. Thus, shortly after illumination a 1:3 ratio of S_0 to S_1 is present. After a prolonged period of dark incubation, however, the S_1-state population is essentially 100% (Hanssum et al., 1985).

There have been conflicting reports in the literature concerning the effect of the 17- and 23-kDa polypeptides, Ca^{2+}, and Cl^- on S-state advancement. The ultraviolet absorbance change measurements of Dekker et al. (1984) have suggested that NaCl-treated PSII membranes were capable of S-state advancement, although with altered kinetics. Ono and Inoue (1985) performed thermoluminescence experiments on PSII membranes that were depleted of the 17- and 23-kDa polypeptides and found that recombination between S_2 and S_3 and Q_A^- occurred normally upon removal of the 17- and 23-kDa polypeptides. More recently, Boussac et al. (1985) have presented luminescence evidence that, in the absence of the 17- and 23-kDa polypeptides, only the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition is blocked. Blough and Sauer (1984), Jansson et al. (1984), and Franzén et al. (1985), however, have reported that

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[1] Abbreviations: chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; kDa, kilodalton; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, O_2-evolving center; P680, primary electron donor in PSII; PSII, photosystem II; Q_A and Q_B, primary and secondary quinone electron acceptors in PSII; Tris, tris(hydroxymethyl)aminomethane.

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the S_2-state multiline EPR signal was suppressed in NaCl-washed PSII preparations. Dismukes and Mathis (1984) observed that an absorption band in the near-infrared spectrum of PSII membranes, which was optimally generated in the S_2 and S_3 states, was not present in PSII samples that lacked the 17- and 23-kDa polypeptides. The inability to detect spectroscopic signals from the S_2 state in NaCl-washed PSII membranes suggested to these authors that either a blockage of the $S_1 \rightarrow S_2$ transition or an alteration of the structure of the OEC resulted from the loss of the 17- and 23-kDa polypeptides.

The effect of Ca^{2+} on S-state transitions in NaCl-washed PSII membranes has been studied. Dismukes and Mathis (1984) found that the near-infrared absorption band, which was suppressed upon treatment of PSII membranes with NaCl, could be photoinduced again in the same samples if 10 mM $CaCl_2$ was added. They reasoned that a stable S_2 state could be produced in NaCl-washed samples only if Ca^{2+} was present. On the other hand, Boussac et al. (1985) observed that adding Ca^{2+} restored the $S_3 \rightarrow S_0$ transition, whereas the $S_1 \rightarrow S_2$ transition was never affected by the removal of the 17- and 23-kDa polypeptides.

The role of Cl^- in O_2 evolution remains a mystery, despite extensive investigation [for a review, see Critchley (1985)]. It is known that the binding of Cl^- to PSII membranes is enhanced by the presence of the 17- and 23-kDa polypeptides (Murata et al., 1983; Andersson et al., 1984). Franzén et al. (1985) observed that depletion of Cl^- from PSII membranes had no effect on the yield of the S_2-state multiline EPR signal. It has also been observed, however, that the depletion of Cl^- from PSII membranes that lacked the 17- and 23-kDa polypeptides resulted in the suppression of the S_2-state multiline EPR signal (Toyoshima et al., 1984; Imaoka et al., 1986). There is also evidence that the absence of Cl^- affects the $S_2 \rightarrow S_3$ (Theg et al., 1984; Itoh et al., 1984) or $S_3 \rightarrow (S_4) \rightarrow S_0$ (Sinclair, 1984) transitions but not the $S_1 \rightarrow S_2$ transition.

Apart from the intermediate S_i states, other photochemically active components of PSII have been investigated. Of these, cytochrome b_{559} has received much attention, partly due to its somewhat enigmatic role in O_2 evolution. Cytochrome b_{559} can exist in two forms that differ in their midpoint potentials. The so-called high-potential form (E_m = 380 mV) of cytochrome b_{559} is reduced by hydroquinone and has been implicated in the mechanism of O_2 evolution (Cramer & Whitmarsh, 1977). By contrast, the low-potential form (E_m = 80 mV) can be reduced by moderately good reductants, such as ascorbate. It has been reported that high-potential cytochrome b_{559} is converted to its low-potential form upon treatments that remove the 17- and 23-kDa polypeptides (Ghanotakis et al., 1986) or the 17-, 23-, and 33-kDa polypeptides (Matsuura & Itoh, 1985). Ghanotakis et al. (1986) also found that the addition of $CaCl_2$ restored O_2 evolution activity to NaCl-washed PSII samples but did not cause a conversion of low-potential cytochrome b_{559} to its high-potential form. This result is surprising in light of the considerable body of work that correlates the loss of O_2 evolution activity with a high to low potential change in cytochrome b_{559} (Matsuda & Butler, 1983a,b).

The purpose of this contribution is to characterize the electron transfer events that take place in PSII membranes lacking the 17- and 23-kDa polypeptides in the temperature range of 77–273 K by quantitating the EPR signals of the several electron donor and acceptor species in a one-electron charge separation in PSII. There are substantial changes in electron donation in PSII at cryogenic temperatures upon removal of the 17- and 23-kDa polypeptides, Ca^{2+}, or Cl^-. We find that the main factors governing the ability to observe the S_2-state multiline EPR signal are Ca^{2+} and Cl^-, and that the 17- and 23-kDa polypeptides are not responsible for maintaining the structure of the Mn site of the OEC.

Experimental Procedures

PSII membranes were prepared from market spinach according to Berthold et al. (1981), with the modifications described by Beck et al. (1985). EPR measurements at room temperature of PSII membranes treated with 5 mM $K_3Fe(CN)_6$ indicate that a negligible amount of EPR signal I is present in our preparations. Since EPR signal I arises from oxidized P700, the primary electron donor in photosystem I, we can state that our PSII preparations are largely free of contamination from photosystem I.

All of the following experimental procedures were carried out at 4 °C and under dim green light. Polypeptide depletion of PSII membranes was performed as follows. A pellet of PSII membranes was suspended in resuspension buffer (RB) (20 mM MES/NaOH, 15 mM NaCl, 5 mM $MgCl_2$, 30% ethylene glycol, pH 6.0) containing 2.0 M NaCl to a final [chl] = 1.2 mg/mL and then incubated in the dark with stirring for 45 min. Following centrifugation at 30000*g* for 20 min the pellet was retreated with 2 M NaCl as above, except that the incubation period was reduced to 5 min. The suspension was pelleted, resuspended in RB, repelleted, and resuspended in RB. Depletion of Cl^- from PSII samples lacking the 17- and 23-kDa polypeptides was accomplished by washing twice with RB in which 15 mM Na_2SO_4 and 5 mM $MgSO_4$ were substituted for 15 mM NaCl and 5 mM $MgCl_2$, respectively. In order to deplete 2 M NaCl-washed PSII membranes of bound Ca^{2+}, samples were pelleted, resuspended to [chl] = 1.0 mg/mL, and incubated for 45 min in RB containing 5 mM EGTA. After centrifugation, the pellet was resuspended in RB containing 5 mM EGTA, repelleted, and resuspended in RB containing 5 mM EGTA. For samples where both Ca^{2+} and Cl^- were depleted, the procedures just described for Ca^{2+} depletion were carried out in a buffer containing sulfate salts instead of chloride salts. In order to reconstitute Ca^{2+} into NaCl/EGTA-treated PSII membranes, samples were washed with RB to remove EGTA and then incubated for 20 min in the dark in a buffer containing 20 mM MES/NaOH, pH 6.0, 100 mM NaCl, and 15 mM $CaCl_2$. After centrifugation, the pellet was washed once more, and finally resuspended, in the same buffer. Washing of PSII membranes with 0.8 M Tris at pH 8.0 to remove the 17-, 23-, and 33-kDa polypeptides and bound Mn was performed according to Yocum et al. (1981).

We have used an EPR method for assaying the fraction of PSII membranes that retained functional Ca^{2+} after the various treatments to remove polypeptides and/or Ca^{2+}. Ghanotakis et al. (1984b) have shown that the depletion of Ca^{2+} from NaCl-washed PSII membranes induced the formation of EPR signal II_f, which can be converted to EPR signal II_{vf} upon Ca^{2+} reconstitution. EPR signal II_f was first detected as a light-induced transient in Tris-washed chloroplasts and probably arises from Z^+, the endogenous primary electron donor to $P680^+$, in samples incapable of water oxidation (Babcock & Sauer, 1975). It appears, therefore, that the generation of EPR signal II_f can be used as a tool for quantitating the amount of functional Ca^{2+} that is released from NaCl-washed PSII membranes as a result of incubation with 5 mM EGTA. Caution must be used, however, in using EPR signal II_f as an assay for functional Ca^{2+} because any treatment that inhibits water oxidation, including Cl^- depletion

Table I: Effect of Polypeptide Release on O_2 Evolution Activity and S_2-State Multiline EPR Signals from PSII Membranes

preparation	O_2 evolution act.[a] (% of control)	S_2-state multiline EPR signal intensity[b] (% of control, 200 K illumination)
control PSII	100	100
2 M NaCl-washed PSII		
(a) 25 mM NaCl	34	70
(b) 25 mM NaCl + 25 mM Ca^{2+}	71	70
(c) 25 mM NaCl, 5 mM EGTA treatment	28	40
(d) as in (c) but with 15 mM $CaCl_2$ and 100 mM NaCl added	80	75
(e) 0 mM NaCl	28	20
(f) 0 mM NaCl, 5 mM EGTA treatment	≤10	0

[a] The control activity for PSII membranes was 440 μmol of O_2/(mg of chl·h) when assayed with 2 mM Ca^{2+}. Without added Ca^{2+}, the activity for PSII membranes was 370 μmol of O_2/(mg of chl·h). [b] Replicate measurements give an estimate of accuracy of ±10%.

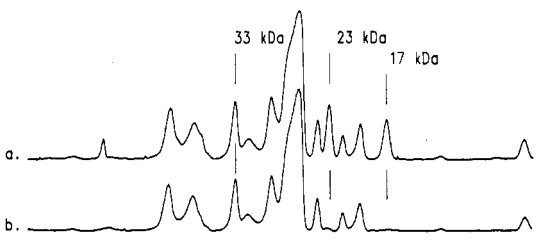


FIGURE 1: Gel densitometer traces of PSII membranes: (a) untreated PSII membranes; (b) PSII membranes treated with 2 M NaCl.

or addition of NH_3, induces EPR signal II_f (Sandusky & Yocum, 1983). The EPR experiments were conducted as follows. Samples were preilluminated at 25 °C for 2 min in order to oxidize the EPR signal II_s species. The light was turned off, and an EPR spectrum was acquired in darkness. Samples were then illuminated in the EPR cavity at 25 °C while their spectra were being acquired. The intensity of EPR signal II_f obtained in NaCl-washed PSII membranes was compared to the intensity of EPR signal II_f obtained in a Tris-washed PSII sample, where 100% of the EPR signal II_f species is oxidized upon illumination. In this method of quantitation we assume, therefore, that the rate of rereduction of the EPR signal II_f species is sufficiently slow in NaCl-washed PSII membranes depleted of Ca^{2+} so as to not limit its complete photooxidation.

The methods outlined by de Paula et al. (1985) were used to perform the EPR measurements and the quantitation of the different EPR signals. The EPR samples contained 3–6 mg of chl/mL. O_2 evolution measurements were carried out as prescribed by Beck et al. (1985) under light-saturating conditions, and electrophoresis through a 12.5% polyacrylamide gel was performed according to Chua (1980), with the modifications introduced by Ikeuchi et al. (1985). The samples were denatured for 20 min at room temperature, and the gels were scanned with a Bio-Rad Model 1650 scanning densitometer in transmittance mode.

RESULTS

Polypeptide Depletion. Figure 1 shows that, as a result of exposure of PSII membranes to 2 M NaCl, the 17- and 23-kDa polypeptides of PSII were extracted. Integration of the gel densitometer peaks corresponding to the 17- and 23-kDa polypeptides (denoted in Figure 1) revealed that at least 90% of each polypeptide was depleted and that all other peaks arising from PSII components were not substantially affected by the NaCl treatment.

Table I shows the effect of depletion of the 17- and 23-kDa polypeptides on the O_2 evolution activity of PSII membranes. It is noted that our preparations show only a slight Cl^- requirement for O_2 evolution, whereas a Ca^{2+} reactivation effect similar to that reported by Ghanotakis et al. (1984b) was

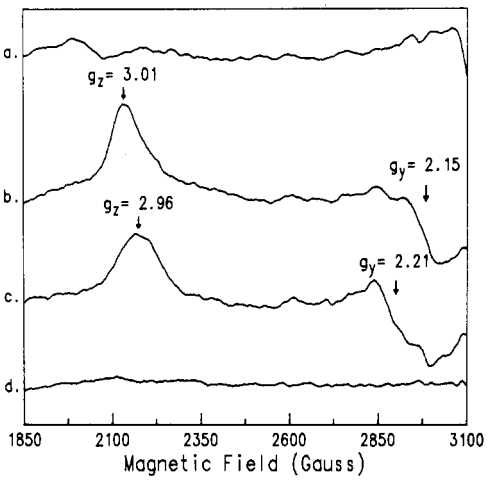


FIGURE 2: Effect of depletion of the 17- and 23-kDa polypeptides of PSII on cytochrome b_{559}, as monitored by EPR: (a) dark-adapted, untreated PSII membranes; (b) 77 K illuminated (10 min) minus dark-adapted, untreated PSII membranes; (c) dark-adapted, NaCl-washed PSII membranes; (d) 77 K illuminated (10 min) minus dark-adapted, NaCl-washed PSII membranes. Instrumental conditions: microwave frequency, 9.1 GHz; microwave power, 0.2 mW; modulation frequency, 100 KHz; modulation amplitude, 20 G; sample temperature, 10 K.

observed. Incubation of NaCl-treated PSII membranes with 5 mM EGTA substantially lowered the O_2 evolution activity especially in the absence of Cl^-. Since the assay medium contained no Ca^{2+}, this result shows that our NaCl-washed PSII preparations have a considerable amount of bound Ca^{2+}.

Cytochrome b_{559}. The EPR spectrum of oxidized cytochrome b_{559} is a very diagnostic tool in distinguishing between its high and low potential forms. Bergström and Vänngård (1982) have shown that oxidized high-potential cytochrome b_{559} exhibits turning points at g_z = 3.08, g_y = 2.16, and g_x = 1.36. The value of g_x quoted here was calculated from the relation $g_{xx}^2 + g_{yy}^2 + g_{zz}^2 = 16$ (Taylor, 1977), since the g_x turning point is broad and cannot be observed in thylakoid or PSII membranes. The cytochrome b_{559} protein from spinach has been isolated and the EPR spectrum of its oxidized low-potential form was observed to have turning points at g_z = 2.93, g_y = 2.26, and g_x = 1.55 (Babcock et al., 1985).

As shown in Figure 2a cytochrome b_{559} is largely reduced in dark-adapted, untreated PSII membranes. It has been shown (de Paula et al., 1985) that illumination at 77 K for 10 min photooxidizes one molecule of cytochrome b_{559} per PSII reaction center. The EPR spectrum displayed in Figure 2b suggests that the high-potential form of cytochrome b_{559} was photooxidized, since the turning points appear at g_z = 3.01 and g_y = 2.15. Depletion of the 17- and 23-kDa polypeptides by NaCl treatment, however, causes the EPR spectrum of dark-adapted samples to exhibit turning points at g_z = 2.96

and $g_y = 2.21$, which arise from oxidized low-potential cytochrome b_{559} (Figure 2c). Quantitation of this EPR signal using myoglobin azide as a spin standard [see de Paula et al. (1985) and Aasa and Vänngård (1975)] showed that 1.7 ± 0.2 molecules of low-potential cytochrome b_{559} were present (average of three measurements). Upon illumination at 77 K for 15 min or treatment with 2 mM K_2IrCl_6, no contributions from oxidized high- or low-potential cytochrome b_{559} were observed (Figure 2d). Since our PSII preparations contain two hemes from cytochrome b_{559} per PSII reaction center (de Paula et al., 1985), it is reasonable to conclude that dark-adapted NaCl-washed PSII membranes contain only the low-potential form of cytochrome b_{559} and that it is completely oxidized in the dark.

The oxidation state, potential form, and amount of cytochrome b_{559} observed by EPR spectroscopy in NaCl-washed PSII membranes were independent of the presence of Ca^{2+} and Cl^-. The samples investigated (Ca^{2+}-depleted; Ca^{2+}-reconstituted, with 100 mM NaCl present; Cl^--depleted; Ca^{2+}- and Cl^--depleted; and 25 mM Ca^{2+} added) all showed the same behavior exemplified by Figure 2c,d.

One Electron Transfer Events. Illumination of PSII membranes that were treated with 75 μM DCMU results in a single stable charge separation since DCMU blocks the transfer of electrons from Q_A to Q_B (Joliot & Kok, 1975). Our study of the one electron transfer reactions in untreated PSII membranes (de Paula et al., 1985) allowed us to determine three distinct electron donation pathways to $P680^+$. At illumination temperatures between 77 and 130 K, high-potential cytochrome b_{559} was found to donate an electron to $P680^+$ in untreated PSII membranes. Between 130 and 220 K, the S_1 state was photooxidized to the S_2 state, which was observed to have two spectroscopic forms. At 130 K, the S_2 state exhibited an EPR signal, which was characterized by a broad turning point at $g = 4.1$. Upon dark incubation at 200 K, this EPR signal was converted into the multiline EPR signal centered at $g = 2.0$. Both the $g = 4.1$ and multiline EPR signals arise from the same Mn-containing site in the S_2 state (de Paula et al., 1985, 1986). Illumination at temperatures higher than 200 K generated the S_2-state multiline EPR signal exclusively. A third mode of electron donation to $P680^+$ was observed in PSII membranes where cytochrome b_{559} was chemically oxidized. Illumination of these samples at 77 K generated an EPR signal with a turning point at $g = 2.0024$, which was tentatively assigned to a chlorophyll cation radical.

Depletion of the 17- and 23-kDa polypeptides from PSII membranes substantially altered the pattern of electron transfer to $P680^+$. The data in Figures 2–5 pertain to NaCl-washed PSII samples where no steps were taken to release bound Ca^{2+}, and which were treated with 75 μM DCMU and resuspended in a buffer containing a total of 25 mM Cl^-.

Illumination of NaCl-washed PSII membranes at 77 K induced the formation of the chlorophyll cation radical centered at $g = 2.0024$ (Figure 3d). Double integration revealed that this EPR signal accounts for essentially 100% of the reaction center concentration. It is obvious from the data presented in Figures 2 and 3 that chl instead of cytochrome b_{559} donated an electron to $P680^+$ at 77 K because low-potential cytochrome b_{559}, present in NaCl-washed PSII membranes, was already fully oxidized.

As the temperature of illumination was raised to 130 K, the S_2-state $g = 4.1$ EPR signal was not generated (Figure 4b). Dark incubation of this sample at 200 K for 2 min did not yield the S_2-state multiline EPR signal as in untreated samples.

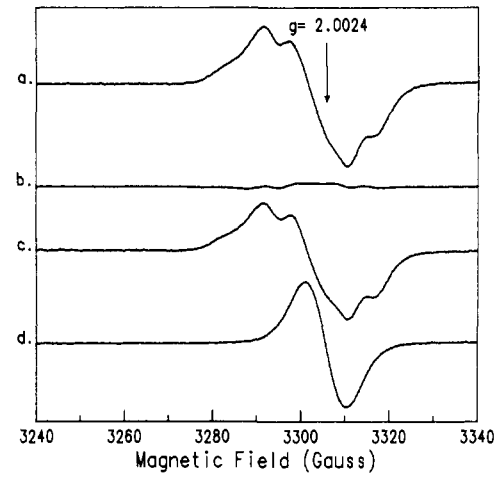


FIGURE 3: Effect of illumination at 77 K on the $g = 2.0$ region of the EPR spectrum of dark-adapted PSII membranes treated with 75 μM DCMU: (a) dark-adapted, untreated PSII membranes; (b) 77 K illuminated (10 min) minus dark-adapted, untreated PSII membranes; (c) dark-adapted, NaCl-washed PSII membranes; (d) 77 K illuminated (10 min) minus dark-adapted, NaCl-washed PSII membranes. Instrument conditions were as in Figure 2, except that the modulation amplitude was 4 G and the sample temperature was 100 K.

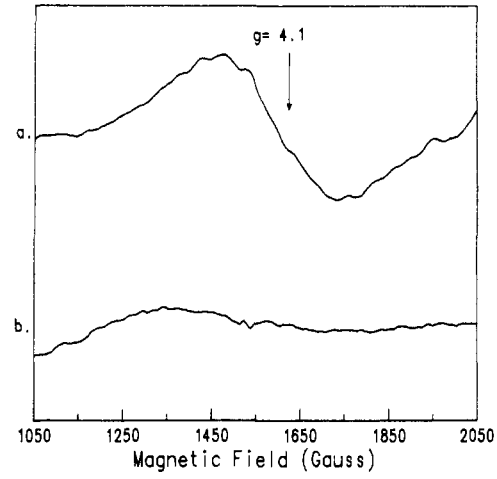


FIGURE 4: Effect of illumination at 130 K on the EPR spectrum of dark-adapted PSII membranes treated with 75 μM DCMU, presented as the difference spectra between the 130 K illuminated and dark-adapted samples: (a) untreated PSII membranes; (b) NaCl-washed PSII membranes. Instrument conditions were as in Figure 2.

Further increase in illumination temperature to 200 K did produce, however, the S_2-state multiline EPR signal (Figure 5b). A comparison of parts a and b of Figure 5 shows that the S_2-state multiline EPR signals obtained from NaCl-washed PSII samples exhibit no differences in ^{55}Mn nuclear hyperfine splittings when compared to untreated samples.

The yields of the EPR signals arising from the oxidized donor species in the samples described above are presented in Figure 6. It is important to ensure that, in the temperature range studied (77–273 K), a single stable charge separation occurred. The extent of charge separation can be determined by monitoring the intensity of the EPR signal due to Q_A^-. A broad EPR signal centered at $g = 1.9$ (observable in Figure 5a,b) arises from Q_A^-, which is magnetically coupled to a Fe(II) ion (Rutherford & Zimmermann, 1984). In all samples investigated, the yield of the Fe(II)-Q_A^- EPR signal remained constant at approximately 95–100% of its maximum intensity throughout the temperature range of 77–273 K (data not shown). This result shows that the phenomena to be described

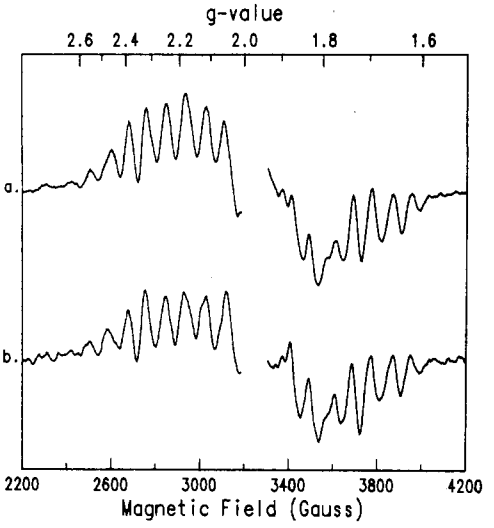


FIGURE 5: Effect of illumination at 200 K on the EPR spectrum of dark-adapted PSII membranes treated with 75 μM DCMU, presented as the difference spectra between the 200 K illuminated and dark-adapted samples: (a) untreated PSII membranes; (b) NaCl-washed PSII membranes. Instrument conditions were as in Figure 2.

were due to a single stable charge separation in PSII.

The intensity of the S_2-state multiline EPR signal is plotted in Figure 6a as the percentage of the maximum intensity of the S_2-state multiline EPR signal observed in control, untreated PSII membranes. It is seen in Figure 6a, therefore, that a maximum of 70% of the control S_2-state multiline EPR signal intensity was attained in samples where only the 17- and 23-kDa polypeptides were depleted. We have also investigated NaCl-washed PSII membranes from wheat seedlings, which were kindly provided by George Cheniae (Radmer et al., 1986). These samples showed 93% of the control intensity of the S_2-state multiline EPR signal when illuminated at 200 K. These results contradict the data obtained by Blough and Sauer (1984), Jansson et al. (1984), and Franzén et al. (1985), who observed a large decrease in the intensity of the S_2-state multiline EPR signal upon removal of the 17- and 23-kDa polypeptides.

The data of Figure 6b also make it clear that a chl molecule was the alternate donor to $P680^+$ under conditions where photooxidation of the S_1 state was prevented. In fact, the inability to observe the S_2-state $g = 4.1$ EPR signal between 100–160 K may indicate that the chl molecule donates an electron much more efficiently to $P680^+$ than the S_1 state over the temperature range in which the $g = 4.1$ EPR signal species is stable (130–160 K). Note that the temperature for half-maximal formation of the S_2-state multiline EPR signal is more than 20 K higher in NaCl-washed PSII samples relative to untreated samples.

It is puzzling, however, that the intensity of the chl cation radical EPR signal should decrease so abruptly at illumination temperatures greater than 200 K, whereas the intensity of the S_2-state multiline EPR signal remained constant in that same range. We have studied, therefore, the stability of the chl cation radical at 210 K. Two different experiments were conducted. The data of Figure 7 show the effect of incubation at 210 K of a NaCl-washed PSII sample that was previously illuminated at 77 K (triangles). The photochemically generated radical decayed by more than 50% during 20 min of incubation in the dark at 210 K. Furthermore, Figure 7 also shows the effect of continuous illumination at 210 K on the yield of the radical (squares). The chl cation radical EPR signal decreased in intensity by approximately 50% from its initial intensity after 8 min of illumination. Both observations are consistent with the existence of a mechanism by which the chl cation radical is reduced at temperatures greater than 200 K. Hence, the sharp drop in yield of the radical at temperatures greater than 200 K can be explained by the instability of the chl cation radical.

Calcium Effect. In order to better characterize electron donation in NaCl-washed PSII membranes so that the basis for the discrepancy between our data and those reports of a suppression of the S_2-state multiline EPR signal in NaCl-washed samples could be evaluated, we have investigated the effects that Ca^{2+} and Cl^- have on electron donation in PSII lacking the 17- and 23-kDa polypeptides. Since Ca^{2+} has been found to stimulate O_2 evolution activity, we performed EPR measurements on NaCl-washed PSII membranes in which the

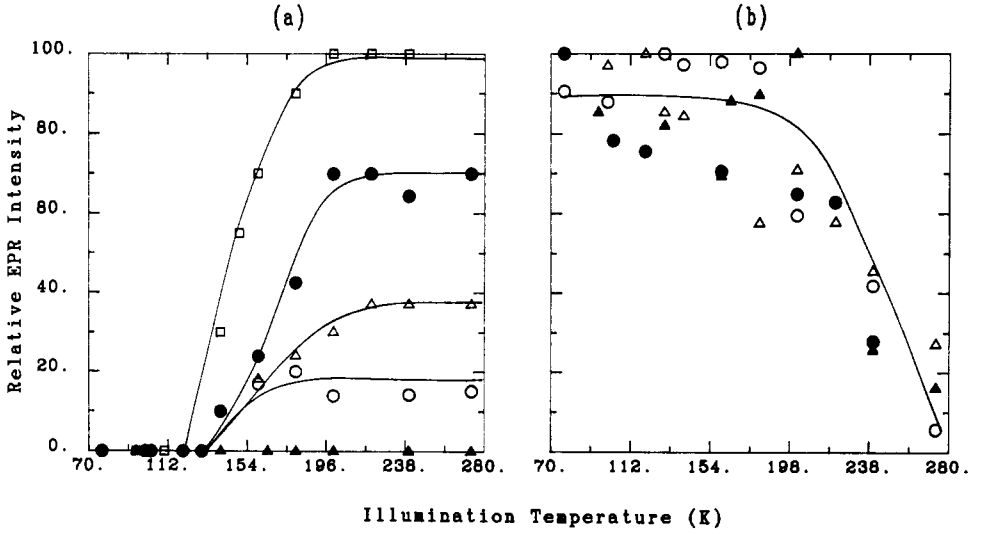


FIGURE 6: Illumination of PSII membranes and its effect on the amplitudes of the (a) S_2-state multiline EPR signal and (b) chl cation radical EPR signal: squares, untreated, control PSII membranes; solid circles, NaCl-washed PSII membranes with Ca^{2+} bound, 25 mM Cl^- present; open triangles, NaCl-washed PSII membranes with Ca^{2+} released, 25 mM Cl^- present; open circles, NaCl-washed PSII membranes with Ca^{2+} bound, no Cl^- added; solid triangles, NaCl-washed PSII membranes with Ca^{2+} released, no Cl^- added. Signal intensities were determined as peak-to-peak heights; for the multiline signal, three peaks to high-field and three peaks to low-field of $g = 2.0$ were added together. The intensities of the chl cation radical EPR signals were normalized with respect to their maximum yields. The intensity of the multiline signal was plotted as the percentage of the maximum intensity of the multiline signal obtained in control, untreated PSII membranes. Estimated uncertainties for the measurement of EPR signals are ± 10%. Instrument conditions were as in Figures 2 and 3.

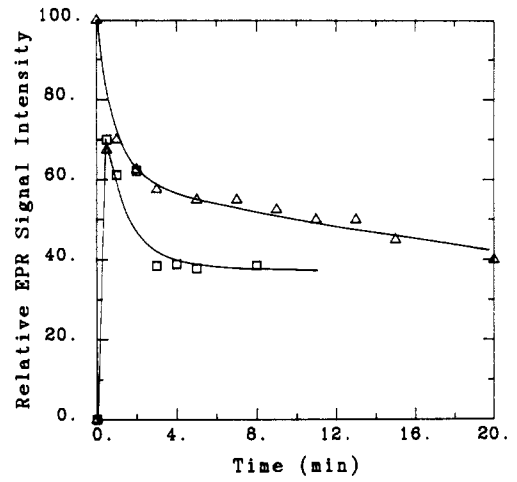


FIGURE 7: Determination of the stability of the chl cation radical at 210 K in NaCl-washed PSII membranes (Ca^{2+} bound, 25 mM Cl^- present): triangles, sample illuminated at 77 K for 10 min then incubated in the dark at 210 K; squares, sample continuously illuminated at 210 K. Data analysis was as in Figure 6; instrument conditions were as in Figure 3.

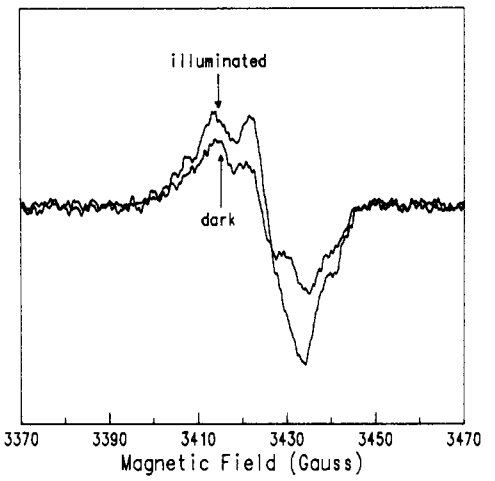


FIGURE 8: Effect of 5 mM EGTA treatment on the room temperature EPR spectrum of NaCl-washed PSII membranes (25 mM Cl^- present). The spectrum labeled as "dark" was obtained after preillumination at 25 °C. Instrument conditions: microwave frequency, 9.45 GHz; microwave power, 2 mW; modulation frequency, 100 KHz; modulation amplitude, 6 G; sample temperature, 25 °C.

concentration of bound Ca^{2+} was varied.

It was noted above that treatment with 5 mM EGTA lowered the O_2 evolution activity rate of NaCl-washed PSII membranes, possibly due to loss of Ca^{2+}. We have assayed the fraction of NaCl-washed PSII membranes that retained Ca^{2+} before and after EGTA treatment. Figure 8 shows that continuous illumination at 25 °C of NaCl-washed PSII membranes treated with 5 mM EGTA induced the formation of EPR signal II_f. The light-induced EPR signal II_f contribution amounted to 75 ± 10% of the intensity of EPR signal II_f detected in a Tris-washed sample under the same conditions. Furthermore, room temperature illumination of a NaCl-washed sample that was not treated with EGTA showed a light-induced EPR signal II_f contribution that amounted to only 16% of the maximum intensity of EPR signal II_f observed in Tris-washed samples. We conclude, therefore, that approximately 75% of bound Ca^{2+} was removed from NaCl-washed PSII samples by EGTA treatment and that approximately 16% of bound Ca^{2+} was lost in NaCl-washed PSII membranes before EGTA treatment.

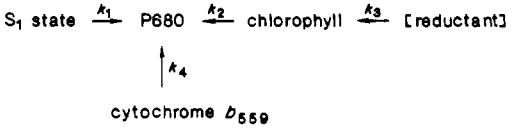
Figure 6a shows that the removal of Ca^{2+} from NaCl-washed PSII membranes decreased the maximum yield of the S_2-state multiline EPR signal to approximately 34% of the control level. Similarly, the O_2 evolution rate of NaCl-washed PSII samples treated with 5 mM EGTA was 28% of the control level (Table I). Moreover, our EPR signal II_f measurements indicate that approximately 25% of the bound Ca^{2+} was not removed by the EGTA treatment. Hence, it is probable that the S_2-state multiline EPR signal being detected is derived from the fraction of NaCl-washed PSII membranes that did not lose their bound Ca^{2+} upon treatment with EGTA. Data obtained on NaCl-washed PSII membranes before EGTA treatment support this conclusion. Our NaCl-washed PSII preparations have lost approximately 16% of the bound Ca^{2+} and exhibit only 70% of the control level of the S_2-state multiline EPR signal. By contrast, PSII preparations from wheat seedlings retain more of their bound Ca^{2+} after NaCl treatment (Callahan & Cheniae, 1985). As was noted above, illumination of these samples at 200 K yielded 93% of the S_2-state multiline EPR signal relative to the control. We conclude that the yield of the S_2 state correlates with the amount of PSII membranes that retains Ca^{2+} and not with the presence of the 17- and 23-kDa polypeptides.

It is important to note that resuspending NaCl-washed samples, which were previously treated with 5 mM EGTA, in a buffer containing 25 mM Ca^{2+} and no EGTA did not affect the yield of the S_2-state mulitline EPR signal and did not suppress the induction of EPR signal II_f. Furthermore, addition of 25 mM Ca^{2+} to NaCl-washed PSII membranes that were not previously treated with EGTA did not increase the yield of the S_2-state multiline EPR signal (Table I). Hence, adding Ca^{2+} to samples in the dark is apparently ineffective in reconstituting Ca^{2+} into NaCl-washed PSII membranes. We have been successful, however, in reconstituting Ca^{2+} into EGTA-treated PSII membranes that lacked the 17- and 23-kDa polypeptides by incubation in a buffer containing both 15 mM $CaCl_2$ and 100 mM NaCl. These samples, when illuminated at 200 K, exhibited an S_2-state multiline EPR signal whose intensity was comparable to that of the S_2-state multiline EPR signal found in NaCl-washed PSII membranes that were never depleted of Ca^{2+} (Table I). It was also found that after readdition of Ca^{2+} in the presence of 100 mM NaCl the total EPR signal II_f intensity was only 8% of that obtained in a Tris-washed PSII sample. It is apparent, therefore, that a relatively high ionic strength is required for the rebinding of functional Ca^{2+} to PSII membranes.

Chloride Effect. The results obtained from NaCl-washed PSII membranes that retained bound Ca^{2+} but were washed with Cl^--free buffer are shown in Figure 6. A rough parallel between the reduction in O_2 evolution rate and maximum yield of the S_2-state multiline EPR signal can be drawn. Similar effects have been reported by Toyoshima et al. (1984) and Imaoka et al. (1986). A more dramatic effect is found upon depletion of both Ca^{2+} and Cl^- from NaCl-washed PSII membranes, which resulted in the complete suppression of the S_2-state multiline EPR signal.

DISCUSSION

The results presented in this contribution allow us to expand the model for electron transfer in PSII which we proposed earlier (de Paula et al., 1985). This model is



In PSII membranes where the 17- and 23-kDa polypeptides and Mn are bound, high-potential cytochrome b_{559} donates an electron to $P680^+$ below 100 K, where k_4 is large relative to k_1 or k_2. Above 200 K, however, S_1 is the preferential electron donor to $P680^+$, thus suggesting that k_1 is large relative to k_2 or k_4 at higher temperatures. The chlorophyll molecule acts as an electron donor to $P680^+$ only at temperatures below 100 K in samples where cytochrome b_{559} is oxidized.

Upon removal of the 17- and 23-kDa polypeptides, cytochrome b_{559} is converted to the low-potential form and is fully autoxidized in the course of dark adaptation, so that the pathway defined by k_4 is prevented. Below 100 K, k_1 is small relative to k_2, and a chlorophyll molecule is photooxidized. The value of k_1 increases with illumination temperature but is never large relative to k_2, so that, even at 200 K, chlorophyll is photooxidized in a substantial fraction of the reaction centers. Above 200 K the chlorophyll cation radical is unstable, however, and it can be reduced by a process characterized by the rate constant k_3. The large value of k_2 relative to k_1 between 130–160 K can explain why the S_2-state $g = 4.1$ EPR signal was not observed in NaCl-washed PSII membranes, since, in that temperature range, the S_1 state simply was not photooxidized. At higher temperatures, the $g = 4.1$ EPR signal species rapidly converts into the multiline EPR signal species. Hence, the suppression of the $g = 4.1$ S_2-state EPR signal does not argue strongly for structural changes in the Mn site as a result of the depletion of the 17- and 23-kDa polypeptides.

The data displayed in Figure 6 indicate that k_1/k_2 is lowered upon removal of Ca^{2+} or Cl^- and is essentially zero when both Ca^{2+} and Cl^- are depleted from NaCl-washed PSII membranes. It is possible that the branching ratio for oxidation of the S_1 state relative to chlorophyll can be modulated by the cofactors Ca^{2+} and Cl^-. There are two ways in which this could occur. A decrease in k_1/k_2 could be explained if Ca^{2+} and/or Cl^- are required for efficient electron transfer from the Mn-containing site to $P680^+$. In this case, the structure of the Mn-containing site itself need not be altered when Ca^{2+} and/or Cl^- are removed.

On the other hand, a decrease in k_1/k_2 in the absence of Ca^{2+} and/or Cl^- could also be explained by invoking structural changes in the Mn site of the OEC. Our data suggest that the absence of the 17- and 23-kDa polypeptides per se does not significantly perturb the environment around the Mn active site of the OEC, since the ^{55}Mn nuclear hyperfine structure of the S_2-state multiline EPR signal was not changed as a result of the NaCl treatment. However, Ca^{2+} and/or Cl^- may be important in maintaining the structural integrity of the Mn site of the OEC. Hence, a second possibility is that depletion of Ca^{2+} and/or Cl^- alters the structure of the Mn site so as to either prevent its photooxidation or produce a structure for the S_2 state that was not observed by EPR spectroscopy.

In addressing the role of Cl^- in the organization of Mn in the OEC, we have to take into account the work of Franzén et al. (1985), who have reported that depletion of Cl^- from untreated PSII membranes did not have a substantial effect on the yield of the S_2-state multiline EPR signal upon 200 K illumination. On the other hand, our results, along with those of Toyoshima et al. (1984) and Imaoka et al. (1986), indicate that depletion of Cl^- from NaCl-washed PSII membranes results in the suppression of the S_2-state multiline EPR signal. In an attempt to reconcile these conflicting data, we can conclude that the inability to observe the S_2-state multiline EPR signal in samples that were depleted of both Cl^- and the 17- and 23-kDa polypeptides indicates that the 17- and 23-kDa polypeptides play an important role in creating a binding site for a special pool of Cl^- ions, which, when bound, render the S_2-state multiline EPR signal observable.

Our studies also allow us to comment on the mode of binding of Ca^{2+} to PSII membranes. Whereas depletion of bound Ca^{2+} by EGTA treatment lowered the yield of the S_2-state multiline EPR signal, addition of 25 mM Ca^{2+} caused no changes in the illumination temperature profile of NaCl-washed PSII membranes, even though Ca^{2+} substantially increased their rates of O_2 evolution (Table I). It is possible, therefore, that once the pool of bound Ca^{2+} is lost, its reconstitution into PSII membranes cannot be achieved by a simple addition of Ca^{2+} in the dark, although such a reconstitution appears to be possible in an O_2 evolution assay. Our results do indicate, however, that the presence of 100 mM NaCl greatly facilitates the rebinding of Ca^{2+} in EGTA-treated PSII membranes that lack the 17- and 23-kDa polypeptides.

Insofar as the release of Ca^{2+} by treatment of PSII membranes with 2 M NaCl may be variable from preparation to preparation and is known to be enhanced in the light (Dekker et al., 1984), loss of bound Ca^{2+} may be responsible for the past failure by other research groups to observe the S_2-state multiline EPR signal in NaCl-washed PSII membranes (Blough & Sauer, 1984; Jansson et al., 1984; Franzén et al., 1985). Furthermore, the failure to produce an S_2-state multiline EPR signal by illumination at 200 K cannot be taken as proof that the $S_1 \rightarrow S_2$ transition is blocked. Such an observation may also be explained by an increase in the temperature for effective oxidation of the S_1 state or by a change in the structure of the S_2 state, such as a conversion to the species that exhibits the $g = 4.1$ EPR signal. Only by a quantitative determination of the extent of a one-electron charge separation and the identification of all possible electron donors can one safely conclude from EPR spectroscopy that the $S_1 \rightarrow S_2$ transition is suppressed by a given treatment.

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Registry No. Ca, 7440-70-2; Cl, 16887-00-6; Mn, 7439-96-5; cytochrome b_{559}, 9044-61-5.

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