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Formation of the S_2 state and structure of the Mn complex in photosystem II lacking the extrinsic 33 kilodalton polypeptide

ANNE-FRANCES MILLER, JULIO C. DE PAULA & GARY W. BRUDVIG
Department of Chemistry, Yale University, New Haven, CT 06511, USA

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Abstract. Electron paramagnetic resonance (EPR) spectroscopy and O_2 evolution assays were performed on photosystem II (PSII) membranes which had been treated with 1 M $CaCl_2$ to release the 17, 23 and 33 kilodalton (kDa) extrinsic polypeptides. Manganese was not released from PSII membranes by this treatment as long as a high concentration of chloride was maintained. We have quantitated the EPR signals of the several electron donors and acceptors of PSII that are photooxidized or reduced in a single stable charge separation over the temperature range of 77 to 240 K. The behavior of the samples was qualitatively similar to that observed in samples depleted of only the 17 and 23 kDa polypeptides (de Paula et al. (1986) Biochemistry **25**, 6487–6494). In both cases, the S_2 state multiline EPR signal was observed in high yield and its formation required bound Ca^{2+}. The lineshape of the S_2 state multiline EPR signal and the magnetic properties of the manganese site were virtually identical to those of untreated PSII membranes. These results suggest that the structure of the manganese site is unaffected by removal of the 33 kDa polypeptide. Nevertheless, in samples lacking the 33 kDa polypeptide a stable charge separation could only be produced in about one half of the reaction centers below 160 K, in contrast to the result obtained in untreated or 17 and 23 kDa polypeptide-depleted PSII membranes. This suggests that one function of the 33 kDa polypeptide is to stabilize conformations of PSII that are active in secondary electron transfer events.

Abbreviations: Chl – chlorophyll; DCBQ – 2,5-dichloro-p-benzoquinone; DCMU – (diuron) 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EGTA – ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; EPR – electron paramagnetic resonance: HSB – high salt buffer; HSCaB – high salt Ca^{2+} buffer; kDa – kilodalton; MES – 2-(N-morpholino)ethanesulfonic acid; P680 – primary electron donor in PSII; PAGE – polyacrylamide gel electrophoresis; PSII – Photosystem II; Q_A – primary quinone electron acceptor in PSII; RB – resuspension buffer; TMPD – N,N,N′,N′-tetramethyl-p- phenylenediamine; Tris – tris(hydroxymethyl)aminomethane; TX100 – Triton X-100; Z – endogenous electron donor to $P680^+$.

Introduction

In light of its pivotal role in water oxidation, the structure and chemistry of the Mn complex of photosystem II are of tremendous interest. Nonetheless, in spite of much recent progress, the identity of the Mn binding protein(s) remains a mystery (for reviews see [14, 17]). A number of candidates have been proposed including the intrinsic 34 kDa polypeptide and the extrinsic 33 kDa polypeptide. Mutants in which a 34 kDa component of the PSII core [23] is

altered have low Mn content and cannot evolve O_2 [7]. The immunologically distinct extrinsic 33 kDa polypeptide [2] has also been correlated with Mn binding because some treatments that release all three extrinsic polypeptides also release Mn, whereas 2 M NaCl treatment to remove only the 17 and 23 kDa polypeptides does not.

Treatments that solubilize all three extrinsic polypeptides (the 17, 23 and 33 kDa polypeptides), but not Mn, such as incubation in 1 M $CaCl_2$, 1 M $MgCl_2$ or 2.6 M urea with 200 mM NaCl, demonstrate that Mn can remain bound in the absence of the 33 kDa polypeptide [15, 24, 27]. Two of the four Mn are not as stably bound in 17, 23 and 33 kDa polypeptide-depleted PSII membranes as in untreated PSII membranes however, and are eventually lost unless the medium contains a high concentration of Cl^- [24]. Thus, the 33 kDa polypeptide helps to stabilize the Mn site and has been postulated to bind to the Mn complex, isolating it from bulk solvent and supplying ligands to Mn (reviewed in [17]). There are two reports which show that the 33 kDa polypeptide can be extracted with Mn bound to it if an oxidizing environment is maintained [1, 35], and that an EPR signal from Mn can be observed from this purified complex [1].

When the three extrinsic polypeptides have been removed, but Mn remains bound to PSII membranes, up to 45% of the O_2-evolving activity of untreated PSII membranes is retained [25]. Thermoluminescence studies of 1 M $CaCl_2$-treated PSII membranes suggest that the S_2 and S_3 states can be attained [29]. Also, the fact that a flash-dependent oscillation of the S_2 state multiline EPR signal intensity, with a period of four and significant intensity after the fifth flash, was observed in 17, 23 and 33 kDa polypeptide-depleted PSII membranes, indicates that none of the S state transitions is blocked [32]. Based on the decay kinetics of EPR signal II_f [16] and TMPD oxidation studies [33], electron transport from the Mn site may be partially inhibited in PSII membranes lacking the 33 kDa polypeptide. The flash dependent yield of O_2 indicates that the $S_3 \rightarrow S_4 \rightarrow S_0$ state transition, in particular, is retarded [26]. These results could explain the low rate of O_2 evolution by samples lacking the extrinsic polypeptides.

Nonetheless, there is disagreement as to whether or not PSII lacking the three extrinsic polypeptides is competent to undergo the $S_1 \rightarrow S_2$ state transition [19, 29]. Removal of only the 17 and 23 kDa polypeptides has been shown not to prevent this transition, or to change the structure of the Mn site in the S_2 state [13], but several attempts to observe the S_2 state by generating the S_2 state multiline EPR signal in PSII membranes lacking the 17, 23 and 33 kDa polypeptides have failed [8, 15, 16, 19] (but see [32]).

We have investigated the $S_1 \rightarrow S_2$ state transition in PSII membranes lacking the 17, 23 and 33 kDa polypepides and have studied the S_2 state multiline EPR signal. We have chosen to monitor the S_2 state multiline EPR signal because it is a sensitive indicator of the Mn complex's structure.

Materials and methods

Except where specified all manipulations were carried out on ice, in dim green light. PSII membranes were prepared using the method in [6] as modified in [5] and stored at 77 K in 30% ethylene glycol resuspension buffer (RB: 15 mM NaCl, 5 mM $MgCl_2$, 30% v/v ethylene glycol, 20 mM MES-NaOH, pH = 6.0) at 4 mg Chl/ml. The $CaCl_2$ treatment was similar to that described in [27]. PSII membranes were diluted to 3 mg Chl/ml with RB, then further with 1.5 M $CaCl_2$ buffer (1.5 M $CaCl_2$, 10 mM NaCl, 30% v/v ethylene glycol, 25 mM MES-NaOH, pH = 6.5) to 1.0 mg Chl/ml and 1.0 M $CaCl_2$, incubated on ice for 30 min and then pelleted at 37,000 × *g* for 30 min. The pellet was resuspended in 1.0 M $CaCl_2$ buffer (same as 1.5 M $CaCl_2$ buffer with 1.0 M $CaCl_2$) to 1 mg Chl/ml and immediately pelleted again to ensure complete removal of the three extrinsic polypeptides. Then the sample was resuspended in high-salt Ca^{2+} buffer (HSCaB: 200 mM NaCl, 15 mM $CaCl_2$, 30% v/v ethylene glycol, 25 mM MES-NaOH pH 6.5) and pelleted three times to decrease the Ca^{2+} concentration to 15 mM.

Prior to Ca^{2+} depletion, samples were resuspended in high-salt buffer (HSB: 200 mM NaCl, 30% v/v ethylene glycol, 25 mM MES-NaOH pH 6.5, adapted from [24]) and pelleted several times until the residual Ca^{2+} concentration was estimated to be less than 1 μM. Ca^{2+} depletion by illumination was achieved by incubating the 17, 23 and 33 kDa polypeptide-depleted sample (in HSB) in room light on ice for 15 min at 0.5 mg Chl/ml. This incubation was repeated once more for complete Ca^{2+} depletion. 1.0 M $CaCl_2$-treated PSII membranes were also depleted of Ca^{2+} in the dark, by incubating in HSB containing 5 mM EGTA for 20 min and then washing away the EGTA with HSB. Ca^{2+} reconstitution was accomplished by resuspending the Ca^{2+}-depleted PSII membranes in 15 mM $CaCl_2$ buffer (HSCaB) or, for reconstitution with Mg^{2+}, in the equivalent 15 mM $MgCl_2$ buffer.

Tris washing was performed as in [36]. O_2 evolution activity was reduced to < 10% of the activity of untreated PSII membranes by this procedure. Heat shock was performed at 1 mg Chl/ml for 5 min at 100 °C. A solution of the 33 kDa polypeptide was obtained by treating extensively 2 M NaCl-washed PSII membranes with 1 M $CaCl_2$ and dialyzing the supernatant against HSCaB. Rebinding of the 33 kDa polypeptide to $CaCl_2$-treated PSII membranes was achieved in the course of a 30 min incubation with a three-fold excess of the polypeptide. The rebinding was monitored with O_2-evolution assays. Polypeptide content was evaluated from peak areas of PAGE densitometric traces. PAGE was performed according to the protocol in [9] with 12.5% acrylamide and 5.5 M Urea (as in [18]).

Chlorophyll assays were performed as in [3] and Mn was quantitated using EPR spectroscopy as in [36]. O_2 evolution activities were assayed as in [5] in the presence of 250 μM DCBQ and 1 mM $K_3Fe(CN)_6$. Untreated PSII membrane preparations had O_2 evolution activities in excess of 500 μmoles O_2/mg Chl/h.

The S_2 state multiline EPR signal was observed at 7 K as described in [11] but

with 100 μM diuron (DCMU) in all the samples. Before illumination each sample was allowed to equilibrate at the chosen illumination temperature in the dark for one minute and then illuminated sufficiently long for the photochemistry to reach its endpoint (4 min at 160 K and above, 10 min at 140 K and below). EPR signal II_s was used as a spin standard [4] to quantitate the Chl cation radical EPR signal. One spin of EPR signal II_s per reaction center was generated by illuminating for 2 min at 0 °C and then freezing in liquid nitrogen. The EPR signal II_s and Chl cation radical signals were recorded immediately as they decay even at 77 K.

Results

The PAGE densitometric traces a and b in Fig. 1 demonstrate that treatment of PSII membranes with 1.0 M $CaCl_2$ releases the extrinsic 17, 23 and 33 kDa polypeptides. Undetectable amounts of the 17 kDa polypeptide and 5 to 20% of the 23 kDa and 33 kDa polypeptides remain associated with 1 M $CaCl_2$-treated PSII membranes. Quantitation of the 23 and 33 kDa polypeptide content in 1 M $CaCl_2$- treated PSII membranes from densitometric traces of Coomassie blue-stained gels is difficult, due to interfering bands (see Fig. 1) and based on O_2 evolution assays (see below), the amount of 23 and 33 kDa polypeptides remaining in 1 M $CaCl_2$-treated PSII membranes is probably less than what is estimated from gels. There has been a report that a 15 kDa fragment of the 33 kDa polypeptide can functionally replace it [34], but neither Tris washing (Fig. 1c), heat shock (data not shown) nor Ca^{2+} depletion (data not shown)

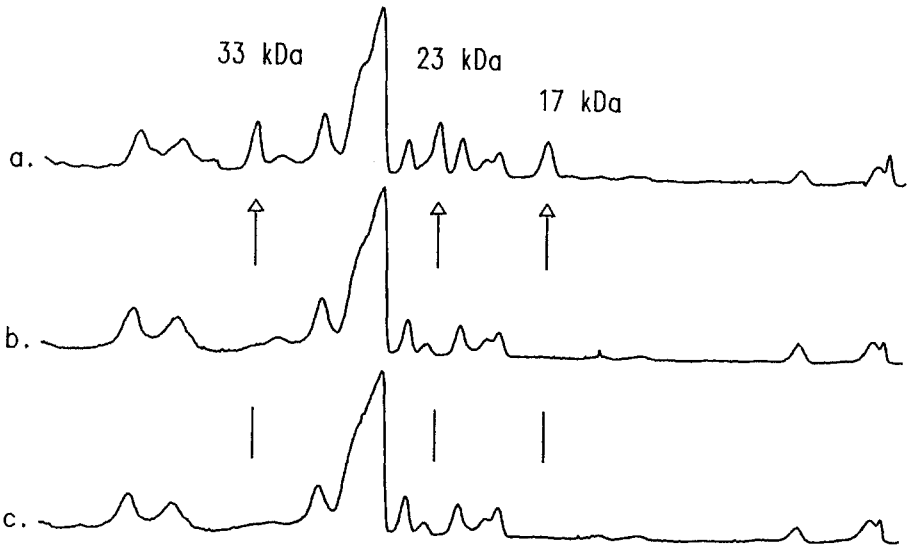

Fig. 1. PAGE densitometry traces of: (a) untreated PSII membranes; (b) 1 M $CaCl_2$-treated PSII membranes; (c) 1 M $CaCl_2$-treated, then Tris washed PSII membranes.

release any proteins from our $CaCl_2$-treated PSII membranes. We do not observe any significant Mn loss from PSII membranes in the course of the treatments described in this paper. Our untreated PSII membranes have 209 Chl/4 Mn (σ = 16.5, average of five measurements on five different preparations) and our 1 M $CaCl_2$-treated PSII membranes have 216 Chl/4 Mn (σ = 37, average of ten measurements on seven different treatments). Although there is sometimes a prominent EPR signal from $Mn(H_2O)_6^{2+}$ in the spectra of $CaCl_2$-treated PSII membranes (Fig. 3a), it represents less than 0.1 Mn per PSII. Therefore, we conclude that our 1.0 M $CaCl_2$-treated PSII membranes are effectively depleted of the extrinsic 17, 23 and 33 kDa polypeptides but retain essentially all of their functional Mn.

The O_2 evolution activity of our 1 M $CaCl_2$-treated PSII membranes as a percentage of that of untreated PSII membranes is 0–10% in the presence of 10 mM NaCl and no added Ca^{2+}, 15–20% in either 200 mM NaCl or 15 mM $CaCl_2$ and 30–45% in the presence of both 200 mM NaCl and 15 mM $CaCl_2$. This behavior agrees well with that reported by other authors for PSII membranes lacking the 17, 23 and 33 kDa polypeptides but retaining functional Mn [19, 21, 25, 28].

We have investigated the photochemistry in 1.0 M $CaCl_2$-treated PSII membranes over the temperature range of 77–240 K. The presence of diuron (DCMU) restricts each PSII reaction center to a single stable charge separation. By comparing these results to the results of similar studies on untreated [11] and 17 and 23 kDa polypeptide-depleted PSII [13], we were able to determine the effects due specifically to removal of the 33 kDa polypeptide.

In untreated PSII membranes continuous illumination produced a quantitative single charge separation throughout the temperature range of 77–220 K [11]. In the course of illumination below 120 K, high-potential cytochrome b_{559} was oxidized. Above 120 K the Mn site was oxidized producing the S_2 state g = 4.1 EPR signal (130 K to 160 K) and the S_2 state multiline EPR signal above 160 K. PSII membranes lacking the 17 and 23 kDa polypeptides also produced the S_2 state multiline EPR signal upon illumination above 160 K, but in slightly reduced yields (80–93% of the yield in untreated PSII membranes) [13]. PSII membranes lacking the 17 and 23 kDa polypeptides did not produce the S_2 state g = 4.1 EPR signal nor was cytochrome b_{559} photooxidized at lower temperatures. Instead, a Chl cation radical ($Chl^{\cdot +}$) was observed and accounted for up to 80% of the sites. In [13], we reported quantitative production of $Chl^{\cdot +}$ following illumination at 77 K and, thus, stable charge separation at all sites at this temperature. However, the yield of $Chl^{\cdot +}$ in this earlier study was determined by using as a spin standard EPR signal II_s prepared the night before and stored at 77 K. We have since determined that only approximately 80% of the EPR signal II_s remains after overnight incubation at 77 K. Thus, the $Chl^{\cdot +}$ yields in [13] should be only 80% of the reported yields. We, therefore, conclude that in the absence of the 17 and 23 kDa polypeptides about 20% of the sites do not form a stable charge separation in the course of illumination at 77 K.

Figure 2 displays the dependence on illumination temperature of the various

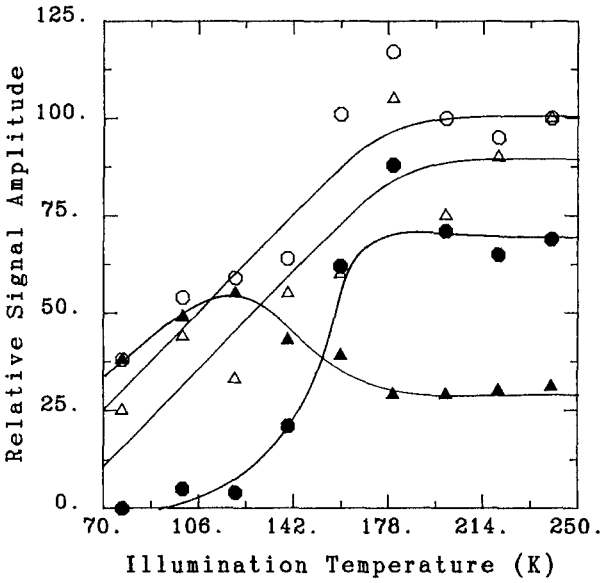

Fig. 2. The effect of illumination temperature on the signal amplitudes of the S_2 state multiline (full circles), Chl radical (full triangles), and $Fe^{2+} \cdot Q_A^-$ (open triangles) EPR signals. The total yield of oxidized electron donors is shown with open circles. Signal intensities were evaluated and scaled as follows: for the amplitude of the S_2 state multiline EPR signal, the peak-to-trough heights of the four large interference-free peaks to high field of g = 2 were added together and plotted relative to the signal amplitude in untreated PSII membranes illuminated at 200 K in the presence of 100 μM DCMU (divron), which arises from 100% of the sites [11]; for the Chl cation radical, the double integral of the EPR signal was plotted relative to the double integral of EPR signal II_s produced at 0 °C, frozen and recorded immediately, which corresponds to one spin per reaction center [4]; for the $Fe^{2+} Q_A^-$ EPR signal, the trough-to-baseline height was plotted relative to that of untreated PSII membranes illuminated at 200 K in the presence of 100 μM DCMU, in which Q_A is reduced in 100% of the sites. The total yield of oxidized electron donors is the sum of the percent Chl oxidation and percent S_2 state multiline EPR signal production at each temperature. Estimated uncertainties in the EPR signal intensities are $\pm$ 10% and in the illumination temperatures $\pm$ 2 K. Instrumental conditions: microwave frequency = 9.1 GHz, microwave power = 0.2 mW, modulation frequency = 100 kHz, modulation amplitude = 20 G, temperature = 7 K. For the Chl radical and EPR signal II_s, the modulation amplitude was 4 G, and the sample temperature was 100 K.

light induced EPR signal intensities in 1.0 M $CaCl_2$-treated PSII membranes. Q_A is the only electron acceptor throughout the temperature range (due to the presence of DCMU) and, hence, the intensity of the $Fe^{2+} \cdot Q_A^-$ EPR signal (open triangles) can be used as a measure of the extent of stable charge separation. Alternatively one can estimate the extent of stable charge separation from the total population of oxidized electron donors (open circles). In 1 M $CaCl_2$-treated PSII membranes, the two species that donate electrons are Chl and the Mn site. Both estimates of the extent of stable charge separation indicate that all of the reaction centers undergo a stable charge separation above approximately 170 K, but that the fraction of PSII supporting a stable charge separation decreases at lower temperatures.

Low-temperature (77 K–120 K) illumination does not oxidize the Mn site and only oxidizes sufficient Chl to account for 35–60% of the sites. The increase in Chl oxidation with increasing illumination temperature in this range parallels the fraction of PSII with a stable charge separation based on the $Fe^{2+} \cdot Q_A^-$ EPR signal. At intermediate temperatures (120 to 180 K), the Mn site and Chl compete for electron donation to $P680^+$ and at illumination temperatures above 180 K the Mn site is the dominant electron donor.

There is good agreement between the intensity of the $Fe^{2+} \cdot Q_A^-$ EPR signal and the total intensity of the EPR signals from Mn sites in the S_2 state and $Chl \cdot^+$ throughout the temperature range. This indicates that Chl and the Mn site account for all the photooxidized sites. At low temperatures we observe fewer sites to be photooxidized, not because we are failing to detect sites in which a different electron donor is photooxidized, but because the fraction of PSII supporting a stable charge separation decreases. Rebinding of the 33 kDa polypeptide to $CaCl_2$-treated PSII membranes increases the $Chl \cdot^+$ yields resulting from 77 K illumination in proportion to the extent of polypeptide recon-

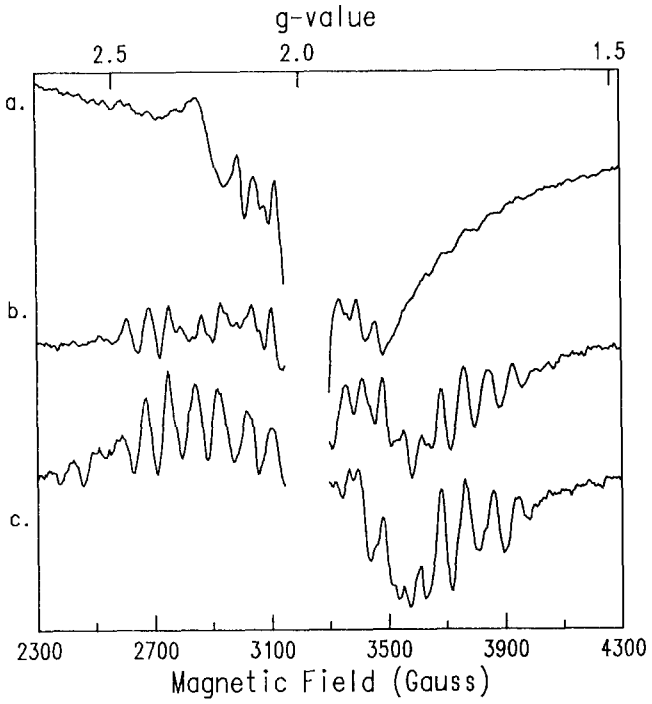

Fig. 3. The effect of illumination at 200 K on the EPR spectrum of dark-adapted PSII membranes in the presence of 100 μM DCMU (diuron). (a) dark spectrum of $CaCl_2$-treated PSII membranes showing the g_y turning point of cytochrome b_{559} at about g = 2.2 and the six hexaaquo Mn lines between approximately g = 2.1 and g = 1.8 that underlie and complicate the S_2 state multiline EPR signal. Illuminated minus dark difference spectra: (b) 1 M $CaCl_2$-treated PSII membranes; (c) untreated PSII membranes. The g = 2 region, with interference from EPR signal II_s, is not shown. Instrument conditions as in the legend of Fig. 2.

stitution. These results suggest that the 33 kDa protein may be important for stable charge separation at cryogenic temperatures.

In contrast to untreated PSII membranes, 1.0 M $CaCl_2$-treated samples do not produce an S_2 state signal at g = 4.1, nor is oxidation of cytochrome b_{559} observed at low temperatures. In both these respects 1 M $CaCl_2$-treated PSII resemble 2 M NaCl-treated PSII [13]. Photooxidation of cytochrome b_{559} is not observed in either case because cytochrome b_{559} is converted to its low-potential form by the $CaCl_2$ treatment and is already oxidized in the dark (data not shown, also see [22]).

Despite depletion of the 33 kDa polypeptide (as well as the 17 and 23 kDa polypeptides), 1 M $CaCl_2$-treated PSII membranes produce a substantial S_2 state multiline EPR signal upon illumination at 200 K (Fig. 3b). The S_2 state multiline EPR signal of the 17, 23, and 33 kDa polypeptide-depleted PSII membranes is 70 $\pm$ 10% as intense as that of the untreated PSII membranes based on the high-field part of the signal. The low-field part gives similar results but is complicated by the underlying turning point of low-potential cytochrome b_{559} at g_y = 2.26 and variable amounts of interference from a small amount of $Mn(H_2O)_6^{2+}$ (Fig. 3a). The differences between the lineshape of the S_2 state multiline EPR signal from untreated PSII membranes and that of the multiline signal from $CaCl_2$-treated PSII membranes are most probably artifacts arising in the subtraction of these two interfering signals. (Note that the only significant differences in the line shape of the two S_2 state multiline EPR signals are in

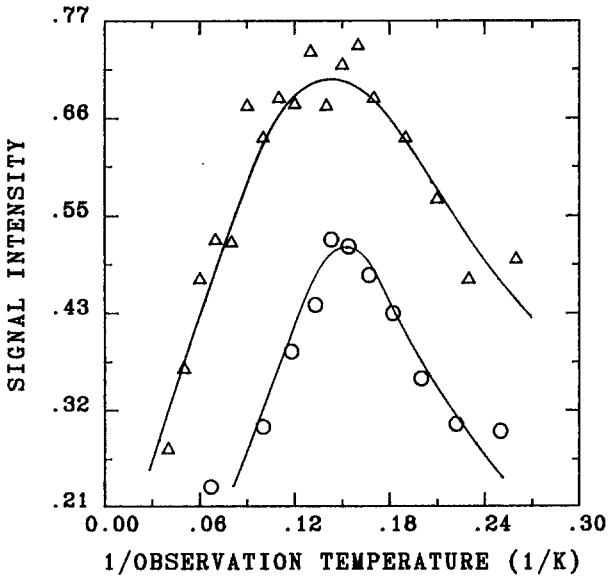

Fig. 4. The dependence of S_2 state multiline EPR signal amplitude on the inverse of the temperature of observation: top, untreated PSII membranes; bottom, 1 M $CaCl_2$-treated PSII membranes. Instrument conditions as in the legend of Fig. 2 except that the microwave power was 0.05 mW in order not to saturate the signal at the lowest temperatures.

regions with interference (compare Figs. 3b and 3c).) Moreover, all the peaks of the S_2 state multiline EPR signal from $CaCl_2$-treated PSII membranes display the characteristic non-Curie temperature dependence of the long-dark-adapted (resting state) S_2 state multiline EPR signal with a maximum at 7 K [12] shown in Fig. 4. From this, we conclude that the structure of the Mn site is not significantly altered in the absence of the 33 kDa protein.

Earlier indications of the importance of Ca^{2+} for production of the S_2 state multiline EPR signal in 17 and 23 kDa polypeptide-depleted PSII membranes [13], coupled with the report [10] that loss of essential Ca^{2+} from polypeptide-depleted PSII membranes is enhanced by illumination, prompted us to search for a Ca^{2+} effect on the $S_1 \rightarrow S_2$ transition in 1 M $CaCl_2$-treated PSII membranes. We did not investigate the possibility of a Cl^- requirement for the $S_1 \rightarrow S_2$ state transition because low-Cl^- media cause inactivation of S-state turnover due to Mn release, but the Cl^- requirement has been recently documented [32].

1.0 M $CaCl_2$-treated samples, depleted of Ca^{2+} by exposure to room light in the absence of Ca^{2+} or by EGTA treatment, show no S_2 state multiline EPR signal (Fig. 5), but the addition of 15 mM Ca^{2+} reconstitutes the S_2 state multiline EPR signal yield to 75%–85% of the initial value (Table 1). The

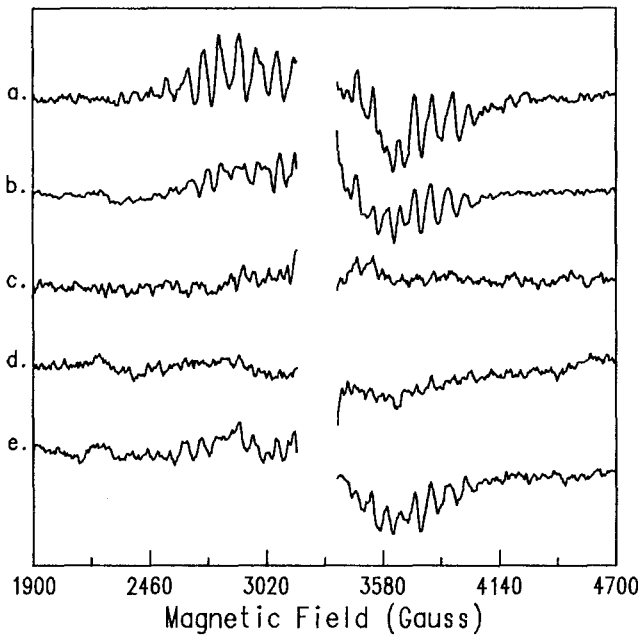

Fig. 5. The effect of Ca^{2+} depletion and reconstitution on the S_2 state multiline EPR signal of 1 M $CaCl_2$-treated PSII membranes illuminated at 200 K in the presence of 100 μM DCMU (diuron). Illuminated minus dark difference spectra: (a) untreated PSII membranes; (b) 1 M $CaCl_2$-treated PSII membranes; (c) 1 M $CaCl_2$-treated PSII membranes depleted of Ca^{2+} by dilution and exposure to room light; (d) 1 M $CaCl_2$-treated PSII membranes depeleted of Ca^{2+} by treatment with EGTA (e) 1 M $CaCl_2$-treated PSII membranes, Ca^{2+}-depleted with EGTA and then reconsituted with 15 mM $CaCl_2$.

Table 1. The effect of Ca^{2+} depletion and reconsitution on S_2 state multiline EPR signal intensity and O_2 evolution activity in 1 M $CaCl_2$-treated PSII membranes.

Sample type	S_2 state EPR signal intensity	O_2 evolution activity
not Ca^{2+} depleted	100%	100%
no Ca^{2+} in medium	60%	35%
exposed to light in 15 mM Ca^{2+} medium	95%	80%
exposed to light in Ca^{2+}-free medium (HS_{hy})	10%	10%
HS_{hy}, with 15 mM Ca^{2+} added back	75%	75%
HS_{hy}, with 15 mM Mg^{2+} added back	25%	25%
EGTA treated	35%	10%
EGTA treated, with 15 mM Ca^{2+} added back	85%	65%

addition of 15 mM Mg^{2+} only reconstitutes the S_2 state multiline signal to a limited extent. Exposure to room light in the presence of 15 mM Ca^{2+} does not alter the amount of S_2 state multiline EPR signal produced, and dilution of Ca^{2+} to $< 1\,\mu M$ in the dark is only partially effective in suppressing the S_2 state multiline EPR signal (60% signal intensity retained). Thus, tightly bound Ca^{2+} is essential for the formation of the S_2 state multiline EPR signal (see also [13]). Our results show that the absence of Ca^{2+} in buffers and EPR media, especially if PSII membranes are exposed to light, could account for a number of the unsuccessful attempts to produce the S_2 state multiline EPR signal in samples lacking the 17, 23 and 33 kDa polypeptides.

Table 1 compares the dependencies of the S_2 state multiline EPR signal intensity and the O_2 evolution rate of 1 M $CaCl_2$-treated PSII membranes on Ca^{2+}. When the two activities are each considered as a percentage of those of 1 M $CaCl_2$-treated PSII membranes not depleted of Ca^{2+}, a strong parallel is evident. These data suggest that the suppression of the S_2 state multiline EPR signal and O_2 evolution may both be symptoms of the same requirement for bound Ca^{2+}. In cyanobacteria Ca^{2+} has been shown to be required for electron transport from Z to P680 [see *e.g.*, 30]. Although our results are consistent with such a role for Ca^{2+}, they do not probe Z; direct evidence [10] suggests that this is not the site of Ca^{2+} action in PSII membranes from spinach.

Discussion

Because not only the 33 kDa polypeptide, but also the 17 and 23 kDa polypeptides are released in the course of treatment with 1.0 M $CaCl_2$, effects due specifically to the loss of the 33 kDa protein should be identified by comparing the results presented in this paper with those of experiments carried out on PSII membranes lacking only the 17 and 23 kDa polypeptides [13]. A number of the

effects of 1 M $CaCl_2$ treatment on low-temperature electron transport also occur in 2 M NaCl-treated PSII membranes [13]. In both cases, cytochrome b_{559} assumes its low-potential form and is oxidized in the dark under the conditions we use, so that neither type of sample displays electron donation from cytochrome b_{559} at low temperatures. The half-maximum temperature of Mn site photooxidation is about 140 K, 20 degrees warmer in both cases than in untreated PSII membranes, with the result that the S_2 state g = 4.1 EPR signal normally produced by 130 K illumination is not observed in either type of polypeptide-depleted PSII preparation. In both 2 M NaCl-treated and 1 M $CaCl_2$-treated PSII membranes, Chl serves as the low-temperature electron donor but not all sites are stably photooxidized below 160 K.

One difference between PSII membranes lacking only the 17 and 23 kDa proteins and PSII membranes lacking the 33 kDa protein as well is that the fraction of centers that produce a stable charge separation following illumination at 77 K decreases from 80% to less than 60%. Apparently the loss of the 33 kDa polypeptide aggravates the inability of polypeptide-depleted PSII membranes to stabilize a charge separation in all of the reaction centers when illuminated at temperatures below 160 K. A similar phenomenon has been observed in PSI; illumination of PSI between 10 and 30 K does not produce a stable charge separation in all of the sites [31].

In the absence of the extrinsic polypeptides, the structure of PSII may not be as well defined as when the polypeptides are bound, and may vacillate between a variety of configurations. At extremely low temperatures motion between most of these states would be frozen out and the resultant heterogeneity of the reaction centers could account for the observation that only a fraction of the sites stabilize charge separations when illuminated at low temperatures. When the illumination occurs at higher temperatures more of the configurations would be able to interconvert with the active one(s) and an increased fraction of the reaction centers would be able to stabilize a charge separation. Thus, one possible role for the extrinsic polypeptides could be stabilization of the configuration(s) of PSII active in secondary electron transfer events (see also [20]).

The features of the S_2 state multiline EPR signal from 1 M $CaCl_2$-treated PSII membranes are very similar to those of the signal from untreated PSII membranes. All the lineshape differences in the low-field portion of the S_2 state multiline EPR signal from polypeptide-depleted PSII membranes can be ascribed to interference from low-potential cytochrome b_{559} and $Mn(H_2O)_6^{2+}$.

The various S_2 state multiline EPR signals from untreated PSII membranes have distinct temperature dependencies that reflect exchange interactions between the Mn ions in the tetranuclear Mn site [12]. The fact that the S_2 state multiline EPR signal from $CaCl_2$-treated PSII membranes has the same temperature dependence as the corresponding signal from untreated PSII membranes indicates that any changes in the site are very minor. Although it is possible to imagine that ligands to Mn once supplied by the 33 kDa polypeptide have been replaced with retention of up to 45% of O_2 evolution activity and an unchanged Mn site structure, it is simpler to conclude from our data that the 33 kDa polypeptide does not supply ligands to Mn but exerts its influence on the

Mn site via structural effects from a binding site somewhere else in the O_2-evolving complex.

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