# The Role of the Extrinsic 33 kDa Protein in $Ca^{2+}$ Binding in Photosystem II†

Andreas Seidler* and A. William Rutherford

*Section de Bioénergétique (CNRS URA 1290), Département de Biologie Cellulaire et Moléculaire, CEA-Saclay, 91191 Gif-sur-Yvette, France*

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ABSTRACT: The role of the 33 kDa protein in $Ca^{2+}$ binding was studied by comparing the EPR properties of photosystem II in the presence and absence of the 33 kDa protein and $Ca^{2+}$. When the removal of the 33 kDa protein was carried out in the dark, a normal manganese multiline EPR signal could be observed when the $S_2$ state was generated. In addition, the split $S_3$ signal could not be generated by illumination at 273 K. Exposure of the 33 kDa protein-less photosystem II to room light did not lead to any change in the EPR properties of the $S_2$ state, but the split $S_3$ state signal at around $g = 2$ could then be generated, indicating that $Ca^{2+}$ was released from this preparation during the exposure to light. Treatment of photosystem II lacking the 33 kDa protein with EGTA in the light led to a modification of the $S_2$ state characterized by a dark-stable multiline EPR signal. Much lower EGTA concentrations were required in order to obtain this modification in the absence of the 33 kDa protein than was required when the 33 kDa protein was present. This indicates that the manganese cluster was more accessible to chelator binding when the 33 kDa protein was absent. When 33 kDa protein-less photosystem II was treated with EGTA in the dark, no modification of the multiline EPR signal of the $S_2$ state of the manganese cluster occurred, nor was $Ca^{2+}$ released as monitored by the inability to generate the split $S_3$ signal. These chelator- and $Ca^{2+}$-binding properties occurring in PSII lacking the 33 kDa protein are very similar to those observed previously in NaCl-washed PSII in which the 33 kDa protein is present (reviewed in Boussac & Rutherford, 1994a). It is concluded that the 33 kDa protein has little or no direct role in binding the $Ca^{2+}$ ion which is required for oxygen evolution.

Photosynthetic oxygen evolution takes place on the lumenal side of photosystem II (PSII)[1] (for recent reviews, see: Rutherford et al., 1992; Debus, 1992). A cluster of four manganese ions is involved in the accumulation of four positive charge equivalents which are generated by four successive charge separations in the reaction center of PSII. After the fourth charge separation, two molecules of water become oxidized and one molecule of oxygen is released. The oxidation states of the manganese cluster are called $S_i$ states, where *i* represents the number of accumulated charge equivalents which varies between 0 and 4.

Three extrinsic proteins with apparent molecular masses of 33, 23, and 16 kDa have been shown to be involved in oxygen evolution (for a recent review, see: Seidler, 1996). Like the manganese cluster, they are located on the lumenal side of PSII. Their function has been widely studied by dissociation and reconstitution experiments. Treatment of PSII with 1 M NaCl removes the 23 and 16 kDa proteins (Åkerlund et al., 1982; Kuwabara & Murata, 1983). When this treatment is carried out in the light, a $Ca^{2+}$ ion is also released (Boussac & Rutherford, 1988a,b; see also: Miyao & Murata, 1986). The oxygen-evolving activity in such preparations is strongly decreased but can be restored by adding 5 mM $Ca^{2+}$ and 30 mM $Cl^-$ to the assay buffer or by rebinding of the 23 kDa protein in the presence of millimolar concentrations of $Ca^{2+}$ and $Cl^-$ (Murata & Miyao, 1985). The rebinding of the 23 kDa protein in the absence of $Ca^{2+}$ did not lead to any reactivation (Ghanotakis et al., 1984). It was shown recently by equilibrium dialysis of intact and NaCl-treated PSII in the presence of $^{45}Ca^{2+}$ that the 23 kDa protein modulates the binding kinetics but not the affinity of $Ca^{2+}$ binding to PSII (Ädelroth et al., 1995).

The effect of $Ca^{2+}$ release on the S states has been studied extensively in NaCl-washed PSII. It was shown that, after release of $Ca^{2+}$ by NaCl treatment in the light, the $S_3$ to $S_0$ transition is inhibited (Boussac et al., 1985; Boussac & Rutherford, 1988a). EPR studies on the $S_2$ state of the manganese cluster which is characterized by a multiline signal did not reveal any changes (Boussac & Rutherford, 1988a; Boussac et al., 1990b). However, in the $S_3$ state a split signal around $g = 2$ appeared which was assigned to an organic radical interacting magnetically with the manganese cluster (Boussac et al., 1989, 1990a). The nature of this organic radical is still under debate. Spectroscopic evidence led to the suggestions that the split signal originates from an oxidized amino acid side chain, either from a histidine or tyrosine residue (Boussac et al., 1990a; Berthomieu & Boussac, 1995; Gilchrist et al., 1995).

If the NaCl treatment was carried out in the presence of millimolar concentrations of $Ca^{2+}$ chelators (e.g., EGTA, EDTA, citrate), the $S_2$ state becomes modified (Boussac et al., 1989; Boussac et al., 1990b). In such preparations, the $S_2$ state is stable at room temperature and the multiline signal

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* Present and correspondence address: Laboratoire de Physiologie Cellulaire Végétale, Département de Biologie Moléculaire et Structurale, CEA, 38054 Grenoble Cedex 9, France.

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[1] Abbreviations: PSII, photosystem II; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone.

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has an altered shape. Studies on $Ca^{2+}$-depleted, chelator-treated PSII by continuous wave and pulsed EPR suggested that the chelator is directly bound to the manganese cluster (Boussac et al., 1990b; Zimmermann et al., 1993; see also: Boussac et al., 1990a). In addition, the modification of the $S_2$ state is lost when such a PSII preparation is depleted of $Cl^-$, and this was explained as being due to the loss of the chelator (van Vliet et al., 1994).

Treatment of PSII with 1 M $CaCl_2$ (Ono & Inoue, 1983, 1984) or 2.6 M urea/200 mM NaCl (Murata & Miyao, 1983, 1984) resulted in the release of all three extrinsic proteins. The manganese cluster is rather unstable after such a treatment. At least 100 mM of $Cl^-$ is required to keep the cluster intact (Murata & Miyao, 1985). Maximal rates of oxygen evolution in the range of 16−45% of the original activity are observed in the presence of 200 mM $Cl^-$ and 10 mM $Ca^{2+}$. The activity could be restored by rebinding of the 33 kDa protein, and at the same time the $Cl^-$ requirement for maximal oxygen evolution was lowered to 30 mM (Ono & Inoue, 1983, 1984; Miyao & Murata, 1984).

In some early reports the multiline EPR signal was not observed in the absence of the 33 kDa protein (Imaoka et al., 1986; Hunziker et al., 1987), but this was most likely due to low $Cl^-$ concentrations (Styring et al., 1987). In the presence of 200 mM $Cl^-$ and 10 mM $Ca^{2+}$ a normal (Miller et al., 1987) or slightly modified (Styring et al., 1987) multiline EPR signal has been observed. However, after treatment of $CaCl_2$-washed PSII with EGTA or by exposure to room light (both treatments were supposed to lead to $Ca^{2+}$ depletion), no multiline EPR signal was observed after illumination at 198 K (Miller et al., 1987). This might indicate that the $S_1-S_2$ transition is blocked or that the $S_1$ state is not stable in such a PSII preparation. Another possibility would be the $S_2$ state did not exhibit any EPR signal in the absence of the 33 kDa protein and $Ca^{2+}$.

Sequence comparison of the 33 kDa protein with known $Ca^{2+}$-binding proteins led to the suggestion that the protein might contain one or more $Ca^{2+}$-binding sites (Coleman & Govindjee, 1987; Wales et al., 1989; Yocum, 1991). It is now generally accepted that there are two $Ca^{2+}$ bound per PSII (for a review of the literature see: Yocum, 1991; see also: Ädelroth et al., 1995). Only one of the two $Ca^{2+}$ is involved in oxygen evolution (Cammarata & Cheniae, 1987; Ono & Inoue, 1988; Han & Katoh, 1992; Ädelroth et al., 1995; see also: Boussac et al., 1985). The other has an unknown function and might be located on an antenna protein, possibly CP29 (Webber & Gray, 1989; Irrgang et al., 1991; Han & Katoh, 1993).

It was found that $Ca^{2+}$ remains essential for oxygen evolution in the absence of the 33 kDa protein (Ono & Inoue, 1983; Miyao & Murata, 1984), indicating that a binding site for $Ca^{2+}$ still exists. However, it is possible that the $Ca^{2+}$-binding site observed in the absence of the 33 kDa protein is modified or is different from that in intact PSII. One possibility which is occasionally discussed is that the native $Ca^{2+}$-binding site is shared between the 33 kDa protein and an intrinsic subunit (see, for example: Yocum, 1991).

The possibility of the involvement of the 33 kDa protein in $Ca^{2+}$ binding was supported by the observation that, in mutants of cyanobacteria where the *psbO* gene encoding the 33 kDa protein has been deleted, $Ca^{2+}$ was absolutely required for photoautotrophic growth of the bacteria, whereas in the wild-type strains depletion of $Ca^{2+}$ from the growth medium had only a moderate effect (Engels et al., 1994; Philbrick et al., 1991). In addition, equilibrium dialysis experiments with isolated PSII seemed to indicate that in the absence of the 33 kDa protein the affinity and/or kinetics of $Ca^{2+}$ binding to PSII are dramatically changed (Ädelroth et al., 1995). In contrast to this study, Chen and Cheniae (1996) reported very recently that PSII lacking the 33 kDa protein still contains a high affinity $Ca^{2+}$-binding site. In this report, we reinvestigated the $Ca^{2+}$-binding properties of PSII lacking the three extrinsic proteins using the appearance of the split $S_3$ EPR signal as measure for $Ca^{2+}$ depletion.

## EXPERIMENTAL PROCEDURES

PSII-enriched membranes were prepared according to Berthold et al. (1981) with the modifications described in Ford and Evans (1983). All further treatments were carried out in the dark or under dim green light unless otherwise stated. The three extrinsic proteins were removed using a procedure similar to that described in Ono and Inoue (1983). Dark-adapted PSII was incubated in a buffer containing 50 mM MES, pH 6.0, 400 mM sucrose, and 1 M $CaCl_2$. After 30 min, PSII membranes were sedimented by centrifugation (48000*g* for 15 min at 4 °C). Then PSII was washed twice (unless stated otherwise) with a buffer containing 50 mM MES, pH 6.0, 400 mM sucrose, and 200 mM NaCl ($SMN_{0.2}$ buffer) in order to lower the $Ca^{2+}$ concentration. $Ca^{2+}$ depletion was carried out by two different methods. Method 1: PSII (0.5 mg/mL chlorophyll) lacking the extrinsic protein was dialyzed against 100 volumes of $SMN_{0.2}$ buffer containing 1 g of Chelex 100/mg of chlorophyll for 1 h at 4 °C. The dialysis was carried out in the dark for the first 40 min, then for 20 min under room light. Method 2: $CaCl_2$-washed PSII (0.5 mg/mL chlorophyll) was exposed to room light at 4 °C for 20 min in the presence of 50 $\mu$M or 10 mM EGTA. $Ca^{2+}$-depleted PSII was sedimented by centrifugation and resuspended in $SMN_{0.2}$ buffer. When $Ca^{2+}$ depletion was carried out in the presence of EGTA, 50 $\mu$M EGTA was included in the resuspension buffer.

Removal of the 23 and 16 kDa proteins from PSII by NaCl-treatment was carried out according to Boussac and Rutherford (1988a).

The 33 kDa protein was overexpressed in *Escherichia coli* and purified as described in Seidler and Michel (1990) except that a linear gradient of 0−200 mM NaCl in 10 mM sodium phosphate, pH 6.5, was used to elute the 33 kDa protein from the DEAE column. For reconstitution of PSII, $CaCl_2$-washed PSII was incubated with a 3-fold molar excess of the 33 kDa protein for 30 min at 273 K in $SMN_{0.2}$ buffer.

For EPR spectroscopy, samples with 3−4 mg/mL chlorophyll were put in calibrated quartz EPR tubes. 1 mM PPBQ was added to the sample in darkness directly before the measurements. EPR spectra were recorded on the dark-adapted samples, after illumination at 198 K (2.5 min) and after illumination at 273 K (2 min) using an 800 W projector lamp. Spectra were recorded at liquid helium temperature with a Bruker ESR200D X-band EPR spectrometer equipped with an Oxford Instruments cryostat.

Oxygen evolution was measured using a Clark-type electrode from Hansatech under saturating white light in $SMN_{0.2}$ buffer containing 1 mM PPBQ.

SDS gel electrophoresis and western blotting were carried out as described in Seidler (1994) except that the proteins


FIGURE 1: Western blot of intact and $CaCl_2$-washed PSII using antibodies against the 33 kDa protein. Lane 1: Intact PSII (4 $\mu$g of chlorophyll = 100%); lane 2: $CaCl_2$-washed PSII (4 $\mu$g of chlorophyll); lanea 3−5: intact PSII (0.6, 0.4, and 0.2 $\mu$g of chlorophyll, corresponding to 15%, 10%, and 5% of the amount loaded in lane 1).

Table 1[a]

| PSII preparation | $Cl^-$ concn (mM) | $O_2$-evolving activity: absolute ($\mu$M $O_2$/mg of chl) | $O_2$-evolving activity: relative (%) |
|---|---|---|---|
| PSII (intact) | 20 | 420 | 100 |
| PSII, NaCl-washed | 30 | 270 | 65 |
| PSII, $CaCl_2$-washed | 200 | 91 | 22 |
| PSII, $CaCl_2$-washed treated with 50 $\mu$M EGTA | 200 | 82 | 20 |
| PSII, $CaCl_2$-washed, 33 kDa protein reconstituted | 30 | 250 | 58 |

[a] All measurements were carried out in 50 mM MES, pH 6.0, 400 mM sucrose, and 10 mM $Ca^{2+}$. $Cl^-$ was added as indicated. The data represent the average of two measurements which were each carried out in triplicate.

were transferred from the SDS/urea gel to the PVDF membrane (Millipore) using the tank blot device from Bio-Rad.

## RESULTS

The three extrinsic proteins were released from PSII by treatment with 1 M $CaCl_2$. The extent of release was controlled by western blotting (Figure 1) and oxygen evolution measurements (Table 1). More than 95% of the 33 kDa protein was released, and the oxygen-evolving activity decreased to 22% compared to untreated PSII (Table 1). In a similar preparation, the loss of activity was shown to be due to a slowdown of the $S_3-S_0$ transition (Miyao et al., 1987). The activity measured in the presence of 30 mM $Cl^-$ and 10 mM $Ca^{2+}$ could be reconstituted to about 60% by rebinding the 33 kDa protein. This is comparable with the activity of NaCl-washed PSII which is about 65%.

The loss in activity of reconstituted compared to intact PSII is mostly due to a slowdown of the electron transfer in PSII. The double reciprocal plot of activity vs light saturation of intact PSII, NaCl-washed PSII, and $CaCl_2$-washed PSII reconstituted with the 33 kDa protein showed straight lines which crossed the *x*-axis at approximately the same point, indicating that the light intensity for half-maximal activity is almost identical for all three PSII preparations (data not shown). The lines for NaCl-washed PSII and $CaCl_2$-washed PSII reconstituted with the 33 kDa protein were superimposable. Their slope was steeper than the slope of untreated PSII, indicating that $V_{max}$ of untreated PSII was higher than for the other two PSII preparations. Only very few reaction centers seem to be irreversibly inhibited due to the release of functional manganese during the $CaCl_2$ treatment and subsequent reconstitution of the 33 kDa protein. Similar behavior has been observed before (Miyao et al., 1987). These data show that the PSII preparation used in the following experiments is largely homogeneous.

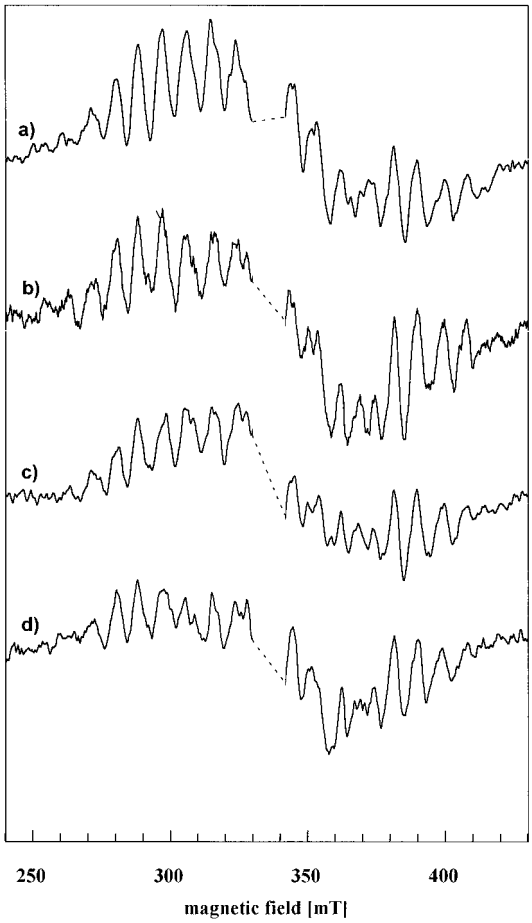


FIGURE 2: Difference EPR spectra of intact and $CaCl_2$-washed PSII. Spectra were recorded before and after illumination at 198 K. Samples were prepared under dimmed green light, filled in EPR tubes (chlorophyll concentration 3−4 mg/mL) and then dark-adapted for 1 h at 273 K before they were stored at 77 K. Before recording EPR spectra, PPBQ was added in absolute darkness to a final concentration of 1 mM. (a) Intact PSII; (b) $CaCl_2$-treated PSII after two $CaCl_2$-free washes prepared under dim green light; (c) as in (b) but $Ca^{2+}$-depleted by exposure to room light for 20 min in the absence of EGTA as described in the Experimental Procedures; (d) as in (b) but before the measurements were carried out, 2 mM EGTA was added in the dark at 273 K and then the sample was incubated for 5 min at the same temperature. Instrument settings: temperature 10 K; microwave frequency 9.44 GHz; modulation amplitude 20 G; microwave power 20 W.

The EPR properties of $CaCl_2$-washed PSII which was kept in the dark or under dim green light during the preparation procedure are shown in Figure 2 (spectrum b). Upon illumination at 198 K, the majority of the reaction centers show a normal multiline EPR signal. The amplitude of this signal was found to be variable among different preparations. The percentage of centers showing the multiline signal could not be strictly correlated with oxygen-evolving activity. We tried to determine the origin of the variability. The control experiments described above showed that the variabilities in EPR signal intensities observed are not due to only partial release in the 33 kDa protein or due to inactivation of the

water-splitting activity in a significant part of the reaction centers.

In order to find out whether the time of incubation of PSII lacking the 33 kDa protein was the origin of the variability of the signal intensity of the multiline EPR signal, we recorded the EPR spectra directly after the $CaCl_2$ treatment and after 6 h of incubation in the dark at 273 K and a chlorophyll concentration of 1 mg/mL in $SMN_{0.2}$ buffer without the addition of $Ca^{2+}$. No difference in the signal intensity was found, indicating that the manganese cluster is stable even in the absence of external $Ca^{2+}$. Even when an identical sample was exposed to room light for 30 min and then further incubated for 5 h in the dark, no change in the amplitude of the signal nor a decrease in activity occurred. However, we found that the number of washes in $SMN_{0.2}$ buffer seem to have a significant effect on the intensity of the multiline EPR signal. When PSII was washed only once after the $CaCl_2$ treatment, the multiline signal was usually 80−90% of the size of the signal of intact PSII. After two washes, 40−80% was found, and after three washes less than 50% was observed. In some preparations no multiline signal could be generated at all, and in those cases the oxygen-evolving activity was slightly lower (10−18%) than usual (22%, see Table 1).

In order to investigate whether the variability in the signal amplitude of the multiline EPR signal was due to the release of $Ca^{2+}$ from a fraction of the PSII reaction centers during the washes, 10 mM $Ca^{2+}$ was included in the buffer which was used for the washings after the removal of the extrinsic proteins. However, no difference in the amplitude of the multiline signal or oxygen-evolving activity (10 mM $Ca^{2+}$ was present during the measurement) could be observed compared to samples which were washed with a $Ca^{2+}$-free buffer. This indicates that the loss of the multiline signal was not due to $Ca^{2+}$ depletion during the washes. At present, we do not know what is the reason for this phenomenon, but it seems that the dilution of PSII itself during the washes causes the loss of signal amplitude. It might be that the $S_1$ state of the manganese cluster is less stable in the absence of the 33 kDa protein and a more reduced state of the manganese cluster is formed. An overreduced manganese complex would not advance to the $S_2$ state upon illumination at 198 K since of the normal S states only the $S_1$ state is photooxidizable at this temperature (Styring & Rutherford, 1988). This view is supported by the observation that in the absence of the 33 kDa protein the manganese cluster becomes very rapidly reduced by external reductants (Tamura & Cheniae, 1985; Tamura et al., 1990). An alternative explanation is that the $S_2$ state of the manganese cluster in a fraction of the reactions centers does not show an EPR signal. The absence of any EPR signal in the $S_2$ state was reported for certain $Cl^-$-depleted PSII preparations (Ono et al., 1986; Boussac & Rutherford, 1994b).

In the following experiments, $Ca^{2+}$-release was monitored by the ability to form the split EPR signal in the modified $S_3$ state of the manganese cluster (equivalent to the $S_2$ state plus an organic radical) after illumination at 273 K. Under these conditions, this signal is taken as being characteristic of inhibition of charge accumulation after the formation of the modified $S_3$ state (Boussac & Rutherford, 1994a).

In order to investigate whether $Ca^{2+}$ was still bound after the $CaCl_2$ treatment and the subsequent two or three $Ca^{2+}$-free washes, the sample was illuminated at 273 K. (N.B.: The term "$Ca^{2+}$-free" in this context means that no $Ca^{2+}$ was added to the buffer. However, commercial sucrose is usually contaminated by $Ca^{2+}$. Due to this contamination we estimated that the $Ca^{2+}$ concentration in the buffer is on the order of 40 $\mu$M.) In NaCl-washed, $Ca^{2+}$-depleted PSII such illumination led to the appearance of a split EPR signal around $g = 2$ which was attributed to the formal $S_3$ state (Boussac et al., 1989). However, no split signal could be observed in $CaCl_2$-treated PSII after two or three $Ca^{2+}$-free washes (not shown), indicating that $Ca^{2+}$ is relatively tightly bound even after the removal of the 33 kDa protein.

In order to release $Ca^{2+}$ from PSII lacking the three extrinsic proteins, $CaCl_2$-washed PSII prepared under dim green light was exposed to room light in the presence and absence of EGTA. In the absence of EGTA, no additional signal could be observed in the dark, but the amplitude of the $Mn^{2+}(H_2O)_6$ signal was slightly increased after the exposure to room light due to some loss of manganese from its active site (Figure 3A, spectrum a). After illumination at 198 K, the normal multiline signal was detected (Figure 2, spectrum c). Further illumination at 273 K led to a disappearance of the multiline signal, and a narrow split signal around $g = 2$ could be observed (Figure 3B, spectrum b), indicating that $Ca^{2+}$ was released from PSII during the exposure to room light. These features are similar to what was observed in NaCl-washed PSII retaining the 33 kDa protein (Boussac et al., 1989). Since exposure to room light of NaCl- or $CaCl_2$-washed PSII induces $Ca^{2+}$ release as observed by the appearance of the split $S_3$ EPR signal, it may seem somewhat surprising that no $Ca^{2+}$ depletion occurred during the strong illumination of NaCl- or $CaCl_2$-washed PSII used to form the modified $S_3$ state. It was suggested earlier for NaCl-washed PSII that under these illumination conditions the S state turnover is too fast to allow $Ca^{2+}$ to be released from PSII (Boussac et al., 1989). This also seems to be the case for $CaCl_2$-washed PSII.

When $CaCl_2$-washed PSII which had been kept in the dark was treated with 2 mM EGTA and subsequently illuminated at 198 K, a normal multiline signal was observed (Figure 2, spectrum d). After thawing of the same sample at 273K and illumination at the same temperature, a small $S_3$-signal could be detected (Figure 3B, spectrum c), indicating the loss of $Ca^{2+}$ in a small fraction of the PSII reaction centers. Illumination of an identical sample directly at 273 K without prior illumination at 198 K did not lead to the appearance of any split $S_3$ signal (not shown), indicating that $Ca^{2+}$ was not released by EGTA in the dark nor by the subsequent illumination. This shows that $Ca^{2+}$ is released from a small fraction of reaction centers during thawing of the sample at 273 K when the manganese cluster was in the $S_2$ state. This is in accordance with what was found for PSII lacking only the 23 and 16 kDa proteins (Boussac et al., 1990a) and demonstrates the tight binding of $Ca^{2+}$ in the $S_1$ state of both PSII preparations.

When the incubation of $CaCl_2$-washed PSII in room light was carried out in the presence of 50 $\mu$M or 10 mM EGTA, a dark-stable multiline EPR signal was observed (Figure 3A, spectra b and c). The amplitude of the dark-stable multiline signal in samples treated with 50 $\mu$M or 10 mM EGTA was identical. This differs from the properties of NaCl-washed PSII which did become modified by 10 mM EGTA but was unmodified by 50 $\mu$M EGTA (Boussac et al., 1990b). However, as in the case of NaCl-washed PSII, the modifica-

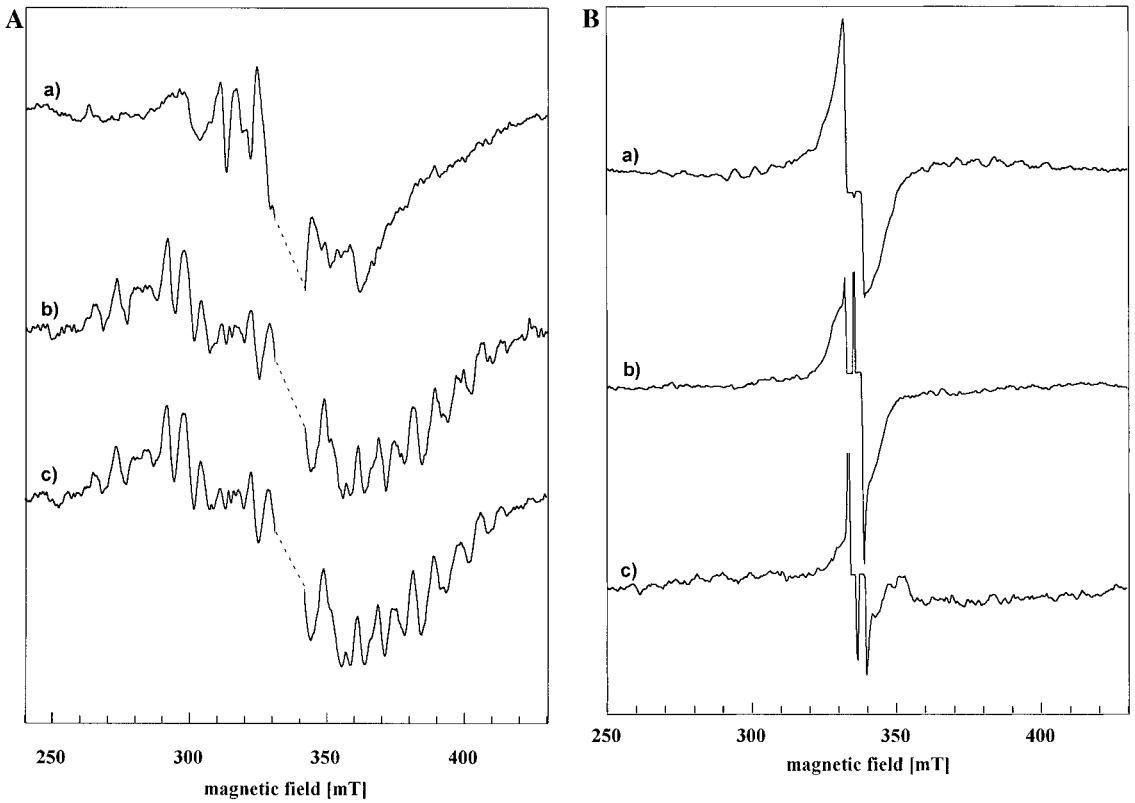


FIGURE 3: EPR spectra of $CaCl_2$-washed PSII exposed to room light in the presence or absence of EGTA. (A) Spectra recorded after addition of 1 mM PPBQ to the samples without prior illumination. (a) $CaCl_2$-treated PSII after two $Ca^{2+}$-free washes prepared under dim green light and then $Ca^{2+}$-depleted by exposure to room light for 20 min during dialysis against $Ca^{2+}$-free buffer containing Chelex X 100 as described in the Experimental Procedures; (b) as in (a) but $Ca^{2+}$-depleted by exposure to room light in the presence of 50 $\mu$M EGTA; (c) as in (a) but $Ca^{2+}$-depleted by exposure to room light in the presence of 10 mM EGTA. (B) Difference EPR spectra of samples recorded before and after illumination at 273 K. (a) $Ca^{2+}$-depleted in the presence of 50 $\mu$M EGTA; (b) $Ca^{2+}$-depleted in the absence EGTA; (c) $CaCl_2$-treated PSII which was supplemented with 2 mM EGTA in the dark, and then illuminated at 198 K (see Figure 2d), then at 273 K. Instrument settings: temperature 10 K; microwave frequency 9.44 GHz; modulation amplitude 20 G; microwave power 20 W.

tion of the manganese cluster by the chelator requires the formation of a higher S state since no modification was observed when the sample was kept in the dark after the addition of EGTA (Figure 2, spectrum d). Further illumination at 273 K led to the disappearance of the multiline signal, and a split signal at $g = 2$ could be observed (Figure 3B, spectrum a). This signal was slightly larger than the one observed when the sample was $Ca^{2+}$-depleted in the absence of EGTA (Figure 3B, spectrum b), indicating that in the latter sample a fraction of reaction centers still contained $Ca^{2+}$.

## DISCUSSION

Previous studies of the role of the 33 kDa protein showed that an $S_2$ manganese multiline EPR signal could be observed in its absence (Styring et al., 1987; Miller et al., 1987). This result was confirmed in the present study, but the extent of reaction centers showing the multiline signal varied drastically depending on the experimental conditions. Interestingly, even when sufficient concentrations of $Ca^{2+}$ and $Cl^-$ were supplied, the size of the signal was found to be not strictly related to oxygen-evolving activity, indicating that the small amplitude of the multiline signal in some preparations did not reflect nonfunctional PSII reaction centers. This variability was shown to be a result of the number of washings of the PSII preparation after the removal of the three extrinsic polypeptides. Possible explanations for this phenomenon are that the manganese cluster is reduced to an S state lower than $S_1$ when such preparations are diluted or that the $S_2$ state in some PSII reaction centers is EPR silent due to a structural perturbation.

When PSII lacking the three extrinsic polypeptides was exposed to room light in a $Ca^{2+}$-free medium, electron transfer was inhibited after the formation of the $S_3$ state as indicated by the appearance of the split $S_3$ EPR signal after illumination at 273 K. This inhibition is attributed to $Ca^{2+}$ depletion because it is reversed by $Ca^{2+}$ addition. In the $Ca^{2+}$-depleted sample, illumination at 198 K results in formation of the normal $S_2$ manganese multiline signal. These results were comparable to those obtained when PSII lacking the 23 and 16 kDa proteins but retaining the 33 kDa protein was exposed to room light in $Ca^{2+}$-free medium containing 50 $\mu$M EGTA (Boussac & Rutherford, 1988a).

The observation of a normal multiline EPR signal in $Ca^{2+}$-depleted PSII lacking the 33 kDa protein is in contrast to the report of Miller et al. (1987) in which this signal could not be generated. The reason for the discrepancy is not clear. However, the ability to form the $S_2$ state by illumination at 198 K may have been lost during or after the $Ca^{2+}$ depletion treatment—probably due to overreduction of the manganese cluster. A similar observation was made in the present study (see above) regardless of the presence or absence of $Ca^{2+}$.

It is known, from studies of PSII lacking only the 23 and 16 kDa proteins, that if $Ca^{2+}$ depletion is carried out in the presence of millimolar concentrations of chelators like EGTA or citrate, the manganese cluster becomes modified. In $Ca^{2+}$-depleted, chelator-treated PSII, the $S_2$ state is stable in the

dark and the multiline EPR signal shows a narrower hyperfine coupling (Boussac et al., 1989). In the present study $Ca^{2+}$ depletion was carried out in the presence of 50 $\mu$M or 10 mM EGTA. Both treatments were equally efficient in creating a modified, dark-stable $S_2$ state, characterized by a modified multiline EPR signal with a narrower hyperfine coupling than the normal multiline signal. The fact that 50 $\mu$M EGTA was sufficient to create the modified $S_2$ state is different from the situation in PSII lacking only the 23 and 16 kDa proteins where this chelator concentration does not modify the manganese complex (Boussac & Rutherford, 1988a). This indicates that in the absence of the 33 kDa protein the manganese cluster is more susceptible to the modification induced by the chelator. This observation is consistent with the idea that the chelator is directly bound to the manganese complex since it was shown that the removal of the 33 kDa protein makes the manganese cluster more accessible to the medium (Tamura & Cheniae, 1985; Tamura et al., 1990; A. Seidler, in preparation).

In the work of Miller et al. (1987) it was reported that no multiline signal could be observed after illumination at 198 K of $CaCl_2$ -washed, EDTA-treated PSII. There are several possibilities which might explain this discrepancy. As mentioned above, the $CaCl_2$-washed PSII might have lost the ability to form a multiline signal during dilution and washing. In this respect, it is of note that the sample of Miller et al. (1987) was washed twice after the EGTA treatment. Alternatively, a fraction of the PSII reaction centers might have exhibited a modified multiline signal which is stable in the dark and which could have been overlooked due to subtraction from the spectrum recorded after illumination. It should be noted that the modified multiline signal has a lower signal amplitude than the normal multiline signal (Boussac et al., 1989).

The tight binding of $Ca^{2+}$ in PSII lacking all three extrinsic proteins observed in the present study apparently contradicts the $Ca^{2+}$-binding studies by Ädelroth et al. (1995). These authors measured $Ca^{2+}$ binding to PSII lacking the 33 kDa protein after incubation with $^{45}Ca^{2+}$ in the dark followed by a wash in $Ca^{2+}$-free buffer. They found only 0.2−0.3 $Ca^{2+}$ bound per PSII under these conditions, and they concluded that the absence of the 33 kDa protein resulted in either a lowered binding affinity or a more rapid exchange of $Ca^{2+}$. However, from the results presented here, it seems that $Ca^{2+}$ is not released in the majority of the PSII reaction centers during dark incubation in a $Ca^{2+}$-free buffer. Therefore, the apparent substoichiometric binding of $Ca^{2+}$ observed by Ädelroth et al. (1995) might be due to only partial (20−30%) exchange of the intrinsic $Ca^{2+}$ with the $Ca^{2+}$ from the medium under the conditions used in their experiments.

The split EPR signal around $g = 2$ observed after illumination at 273 K of PSII lacking the extrinsic proteins and $Ca^{2+}$ shows a narrower splitting than in PSII containing the 33 kDa protein. It resembles in its linewidth the $S_3$ signal observed in $Cl^-$-depleted PSII (Boussac & Rutherford, 1994b). However, it was observed already that in the absence of the 23 and 16 kDa proteins this signal has a narrower splitting than in their presence (Boussac et al., 1990b). This then is a further example of the extrinsic polypeptides modulating the magnetic interaction between the manganese cluster and the organic free radical responsible for the split $S_3$ signal.

As mentioned in the introduction, mutants of the cyanobacterium *Synechocystis* PCC 6803, in which the *psbO* gene encoding the 33 kDa protein has been inactivated, exhibit a more marked requirement for $Ca^{2+}$ in the growth medium than does the wild-type (Philbrick et al., 1991; Engels et al., 1994). Assuming that omission of $Ca^{2+}$ from the growth medium results in a low intramolecular $Ca^{2+}$ concentration, this might be taken as an indication for the 33 kDa protein being directly involved in $Ca^{2+}$ binding. However, in the light of the results of our present work, another interpretation is more likely. In higher plant PSII the 23 kDa protein seems to play an important role in maintaining the $Ca^{2+}$ in or close to its functional site during the S state turnover, preventing the loss of $Ca^{2+}$ in the $S_3$ state in which it is rapidly exchangeable and/or weakly bound. In cyanobacteria, however, the 23 kDa protein is absent (Stewart et al., 1985). It is possible that the role of the 23 kDa protein is in cyanobacteria fulfilled either by another protein which may also rely on the presence of the 33 kDa protein for its binding or by the 33 kDa protein itself. Another possibility is that an elevated $Ca^{2+}$ concentration in the thylakoid lumen protects manganese from becoming overreduced—a similar effect of $Ca^{2+}$ has been shown for plant PSII *in vitro* (Tamura & Cheniae, 1985; Tamura et al., 1990)—or being released from PSII after overreduction by endogenous reductants, as shown for NaCl-washed PSII (Mei & Yocum, 1992).

In the literature there are three main indications for the 33 kDa protein having an effect on $Ca^{2+}$ binding: (1) no multiline EPR signal could be observed after $Ca^{2+}$ depletion from 33 kDa protein-less PSII (see above); (2) $^{45}Ca^{2+}$ is apparently either weakly bound or rapidly exchanged in the absence of the 33 kDa protein (see above); and (3) cyanobacterial mutants with inactive *psbO* gene were unable to grow in the absence of $Ca^{2+}$ in the growth medium (see above). The data presented in this article argue against the first and the second points. Point 3 is not in itself a strong argument for a binding of $Ca^{2+}$ by the 33 kDa protein as discussed above. We conclude then that it is unlikely that the 33 kDa protein is involved in the binding of the $Ca^{2+}$ which is required for oxygen evolution, either directly by providing ligands or indirectly by maintaining the subunit to which $Ca^{2+}$ is bound in its functional conformation. If the 33 kDa protein does bind $Ca^{2+}$ as suggested from the sequence homologies to known $Ca^{2+}$-binding proteins (Coleman & Govindjee, 1987; Wales et al., 1989; Yocum, 1991), then such a $Ca^{2+}$ is not obviously involved in oxygen evolution and has so far escaped detection. Our data are consistent with the very recent finding by Chen and Cheniae (1996) that PSII contains a high affinity $Ca^{2+}$-binding site even in the absence of the 33 kDa protein.

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