Chloride-Depletion Effects in the Calcium-Deficient Oxygen-Evolving Complex of Photosystem II

Pieter van Vliet,* Alain Boussac, and A. William Rutherford

Section de Bioénergétique (URA CNRS 1290), Département de Biologie Cellulaire et Moléculaire, CEA Saclay, 91191 Gif-sur-Yvette, France, and Department of Molecular Physics, Agricultural University, Wageningen, The Netherlands

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ABSTRACT: The effects of Cl^- depletion in photosystem II (PS-II)-enriched membranes have been investigated by electron paramagnetic resonance (EPR) spectroscopy after removal of the 17- and 23-kDa polypeptides and depletion of Ca^{2+} by NaCl treatment. When the salt treatment was done in the presence of a high concentration (5 mM) of the chelator [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), a modified dark-stable multiline signal was observed from the S_2 state and a 13 mT wide S_3 signal could be generated by illumination at 0 °C as reported previously for experiments conducted under these conditions [Boussac, A., Zimmermann, J.-L., & Rutherford, A. W. (1990) *FEBS Lett. 277,* 69–74]. The modified S_2 multiline signal was lost after a further Cl^- depletion in the presence of a low EGTA concentration (50 μM). Upon Cl^- reconstitution, a normal S_2 multiline signal could be generated by continuous illumination at 200 K. In contrast, a lowering of the EGTA concentration (50 μM) alone, in the presence of Cl^- (30 mM), had no effect on the modified S_2 multiline signal. These results indicate that the modification of S_2 is due to binding of the chelator to PS-II and that Cl^- stabilizes the chelator binding. When Cl^- depletion in Ca^{2+}-depleted PS-II was done in the presence of a high concentration of EGTA (5 mM), the modified S_2 multiline signal disappeared but was regenerated by Cl^- reconstitution in darkness. These results indicate that when Cl^- depletion is done to the EGTA-modified PS-II, the S_2 multiline signal disappears but the S_2 state remains stable in the dark. Thus, EGTA binding and Cl^- depletion appear to be additive phenomena. Cl^- depletion also modified the S_3 EPR signal, showing a narrrow signal (<10 mT) around $g = 2$. This modification of the S_3 signal was reversed by Cl^- reconstitution, resulting in the reappearance of the 13 mT wide S_3 signal. The modifications of S_2 and S_3 due to Cl^- depletion observed in Ca^{2+}-depleted membranes are similar to those observed following Cl^- depletion in regular PS-II membranes, in which functional Ca^{2+} is thought to be present. These results, therefore, indicate that the modifications of the S_2 and S_3 EPR signals due to Cl^- depletion are independent of Ca^{2+}. Investigations of PS-II membranes which were salt-treated without EGTA revealed that the chemical 2-(*N*-morpholino)ethanesulfonic acid (MES), generally used as a pH buffer, was able to modify the S_2 state, in a similar fashion to EGTA. In consideration of the components that are known to modify S_2 [EGTA, (ethylenedinitrilo)tetraacetic acid (EDTA), citrate, pyrophosphate, and MES), the results indicate that the modification of S_2 is due to binding of these components, by their anionic groups containing oxygen, near to or on the Mn cluster itself. The observed effects of Ca^{2+} and Cl^- depletion in PS-II may be relevant to the proposed role(s) of Ca^{2+} and Cl^- in controlling substrate binding in the functional charge accumulation cycle.

Photosynthetic water photolysis is catalyzed by the photosystem II protein complex (PS-II)[1] and is thought to occur upon accumulation of four positive charges in a cycle, consisting of five intermediate states designated S_0 to S_4, where the subscript is the number of charges stored (Kok et al., 1970). The kinetic properties of the charge accumulation states under different experimental conditions have been characterized in detail [reviewed in Rutherford et al. (1992) and Debus (1992)]. A manganese cluster, which is thought to consist of four manganese ions, plays a central role in the charge accumulation cycle. Also Ca^{2+} and Cl^- are essential in the mechanism of water photooxidation [for reviews, see Rutherford et al. (1992) and Debus (1992)]. Three extrinsic proteins of 33, 23, and 17 kDa, present at the lumenal side of PS-II, contribute to the stability of the oxygen-evolving enzyme but are not essential for oxygen evolution [reviewed by Murata and Miyao (1985)]. The 33-kDa polypeptide stabilizes the manganese cluster. The 17- and 23-kDa extrinsic polypeptides play a role in retention of functional Ca^{2+} and Cl^- (Murata & Miyao, 1985; Homann, 1988a).

In the study of the role of Ca^{2+} and Cl^- in the mechanism of water photooxidation, a number of methods have been developed to deplete these ions, inducing reversible inhibition of oxygen evolution and retention of the manganese cluster in its site (Homann, 1988a,b; Debus, 1992).

Several studies, using different techniques, have indicated that Cl^- depletion leads to inhibition of the S_3 to S_0 transition [reviewed in Rutherford et al. (1992)]. Depletion of Ca^{2+} involves dissociation of the 17- and 23-kDa proteins by washing of the PS-II membranes in NaCl (Ghanotakis et al., 1984) or treatment at low pH (Ono & Inoue, 1988). In salt-washed PS-II, Ca^{2+} is released most easily in S_3 (Boussac & Rutherford, 1988b), and for this reason Ca^{2+} release is greatly enhanced when the treatments are done in the light (Dekker

* To whom correspondence should be addressed. CEA Saclay. Telephone: 33-1-69-08-29-40. Fax: 33-1-69-08-87-17.

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[1] Abbreviations: PS-II, photosystem II protein complex; Tyr_D, sidepath electron donor of PS-II responsible for EPR signal II_{slow}; Q_A, primary quinone electron acceptor of PS-II; CW, continuous wave; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone.

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et al., 1984; Boussac et al., 1985; Miyao & Murata, 1986; Boussac & Rutherford, 1988a,b). Following Ca^{2+} depletion in PS-II, the S_3 to S_0 transition in the charge accumulation cycle is inhibited (Boussac et al., 1985; Boussac & Rutherford, 1988a). Under physiological conditions, light-dependent Ca^{2+} release from the donor side of PS-II may be involved in regulation of the balance between photosynthetic electron transport and the dark reactions (Krieger et al., 1992).

Many studies on the charge accumulation cycle have been done using EPR spectroscopy. In regular PS-II membranes, a multiline EPR signal at $g = 2$ is observed in the S_2 state (Dismukes & Siderer, 1981). This signal can be generated by illumination treatments allowing for a single stable charge separation, e.g., single-flash illumination at room temperature, or by continuous illumination at 200 K (Dismukes & Siderer, 1981; Hansson & Andréasson, 1982; Brudvig et al., 1983). Under some conditions, the S_2 state also exhibits an EPR signal at $g = 4.1$ [reviewed in Rutherford et al. (1992)]. Following inhibition of oxygen evolution by treatment with SO_4^{2-} at pH 7.5, no S_2 multiline EPR signal was detected after a single flash or illumination at 200 K, and little (Ono et al., 1986) or no (Boussac et al., 1992) $g = 4.1$ EPR signal appeared. However, the multiline EPR signal was restored by addition of Cl^- in the dark, following illumination [Ono et al., 1986; see also Boussac et al. (1992) and Boussac and Rutherford (1994)]. From these observations it was concluded that Cl^- depletion results in a modified S_2 state, resulting in the loss of its characteristic EPR signal, which is reversed to the normal S_2 state by Cl^- reconstitution (Ono et al., 1986; Boussac & Rutherford, 1994).

In functional PS-II, no signals from states other than the S_2 state have been detected by conventional CW-EPR. However, in Ca^{2+}-depleted PS-II, an EPR signal around $g = 2$ was observed, corresponding to a formal S_3 state, with a peak to trough width of 16.4 mT when the 17- and 23-kDa extrinsic polypeptides were reconstituted (Boussac et al., 1989); a 13 mT wide EPR signal was observed in the absence of these polypeptides (Boussac et al., 1990a). Upon generation of this signal from S_2, the multiline EPR signal was completely suppressed. Boussac et al. (1990b) proposed that, upon formation of S_3 from S_2, the oxidation state of the manganese cluster remains unchanged and that an oxidized radical is formed instead. In this model, the organic radical with $S = 1/2$ interacts with the $S = 1/2$ manganese cluster (Boussac et al., 1990b). The radical species was proposed to be a histidine radical on the basis of its absorption spectrum in the ultraviolet (Boussac et al., 1990b), although alternative explanations were not excluded [discussed in Rutherford and Boussac (1992)]. Besides their formation in Ca^{2+}-depleted PS-II, S_3 signals were also observed following inhibition of oxygen evolution by treatments with F^- (Baumgarten et al., 1990), SO_4^{2-} (Boussac et al., 1992), NH_3 (Andréasson & Lindberg, 1992; Hallahan et al., 1992), or acetate (MacLachlan et al., 1993).

In Ca^{2+}-depleted PS-II following a salt wash with millimolar amounts of the chelator EGTA (Boussac et al., 1989) or EDTA (Ono & Inoue, 1990) a dark-stable S_2 modified multiline EPR signal was observed, indicating a perturbation of the manganese cluster due to the treatment. This modification was eliminated by reconstitution of Ca^{2+} (Boussac et al., 1989). A modified multiline EPR signal was also observed following a low-pH treatment in the presence of citrate (Sivaraja et al., 1989). It was shown that the modification of S_2 was induced by millimolar concentrations of the chelator and required light (Boussac et al., 1990a). The chelator-induced modification was discussed as being due to either additional removal of Ca^{2+} by the chelator or direct binding of the chelator to the manganese cluster (Boussac et al., 1990a). The second possibility was favored.

Subsequently, ESEEM spectra were obtained from the modified S_2 (Zimmermann et al., 1993). An ESEEM frequency, probably arising from ^{14}N, was present in ^{15}N-labeled PS-II. This frequency was proposed to arise from the coupling between the manganese cluster and the ^{14}N nucleus from EGTA. This was supported by the observation that this frequency was absent when pyrophosphate was used to induce the modification of S_2. These data were taken as further support for the idea that direct binding of the chelator to PS-II is responsible for the modification of S_2.

Nevertheless, despite these indications, the modification of the multiline EPR signal is frequently considered to be due to Ca^{2+} release. At least in part, this is due to the fact that the evidence and arguments for chelator binding to PS-II are far from conclusive. Thus, the question whether the chelator-induced modification is due to Ca^{2+} removal or to binding of the chelator itself warrants further investigation.

This report deals with the effects of Cl^- depletion on the S_2 and S_3 states in Ca^{2+}-depleted PS-II membranes. It has been suggested that Ca^{2+} and Cl^- may play roles in controlling functional association of the substrate to the active site [reviewed in Rutherford et al. (1992)]. Experimental evidence is presented indicating that chelator binding to PS-II occurs when Ca^{2+} is absent. In addition, Cl^- influences chelator binding.

MATERIALS AND METHODS

Photosystem II-enriched membranes were prepared according to the method of Berthold et al. (1981) with the modifications of Ford and Evans (1983) and were stored at −80 °C until use. The activity of these membranes was ≃500 μM O_2/mg of chlorophyll/h.

Salt treatment of PS-II membranes was done as described by Boussac et al. (1990a) by incubating PS-II membranes (0.5 mg of chlorophyll/mL) for 30 min in 1.2 M NaCl, 0.3 M sucrose, and 25 mM MES, pH 6.5, under room light at 5 °C. This was done either in the presence of EGTA (5 mM) or in the absence of EGTA (see below).

The salt-washed PS-II membranes were spun down, which was followed by a range of different washing procedures on ice under room light as described in the Results. Unless stated otherwise, the solutions were buffered with 10 mM MES (pH 6.5). Ca^{2+}-free buffer solutions were prepared by putting them through a CHELEX-100 column.

Cl^- depletion in salt-washed PS-II was done by three washes (resuspension, dilution, and centrifugation) in Cl^--free solutions at pH 6.5. The rationale behind this treatment was that the 17- and 23-kDa polypeptides, which are important for retaining Cl^- in its functional site (Homann, 1988a), are absent following the salt treatment, and Cl^- depletion in this preparation is therefore considered to occur by additional washes in Cl^--free solutions.

The membranes were resuspended at 8–12 mg of chlorphyll/mL in the final solution used in the washing procedure described in the results and were put in calibrated quartz EPR tubes, dark-adapted on ice for ~1 h, frozen in the dark, and stored in liquid nitrogen until use for EPR measurements. Further additions to these membranes were done in the EPR tube in the dark after thawing. Illumination of the samples was done in the presence of PPBQ dissolved in dimethyl sulfoxide, added as an external electron acceptor to a final

concentration of 1 mM. The samples were illuminated in a non-silvered Dewar flask containing ethanol cooled to 198 K with solid CO_2 or cooled to 0 °C with liquid nitrogen. After the illumination at 0 °C, the samples were rapidly frozen (<1 s) to 198 K and stored in liquid nitrogen. Illumination was done with an 800-W projector through 2 cm of water and an infrared filter.

EPR spectra were recorded at liquid helium temperatures with a Bruker ER 200 D X-band spectrometer equipped with an Oxford Instruments cryostat.

Differences in signal intensity of the EPR spectra, due to variable chlorophyll concentrations in the EPR tubes, were eliminated by scaling relative to the amplitude of $Tyr_D^{\bullet}$ measured at an unsaturating power (80 μW) at 20 K. No significant differences were observed in the amplitude of $Tyr_D^{\bullet}$ in these preparations before and after illumination procedures.

Salt-treated membranes not used for EPR measurements were stored in aliquots at −80 °C and were used afterwards for measurements of oxygen-evolving activity. For these measurements, the membranes were thawed out and resuspended in 30 mM NaCl, 0.5 M sucrose, and 25 mM MES (pH 6.5) to a chlorophyll concentration of ~2.5 mg/mL and stored on ice in darkness. The measurements were done in the buffer solution used for resuspension, using a Clark type electrode, at 20 °C under continuous saturating light. The chlorophyll concentration was 20 μg/mL, and 0.5 mM PPBQ was added as an external electron acceptor.

The oxygen-evolving activity of Ca^{2+}-depleted PS-II membranes used in this study was largely inhibited. The residual activity of the different preparations was lost relatively rapidly with a half-inhibition time of 20 s during the measurement and showed an initial rate of about 30% of that when 6 mM Ca^{2+} was present during the measurement. In the presence of 6 mM Ca^{2+} the loss of activity during the measurement was comparable to that of the intact starting material with a half-inhibition time of 80 s, and the initial rate was about 65% of the control, indicating the presence of a fraction of damaged centers of about 35% following washing and reconstitution treatments.

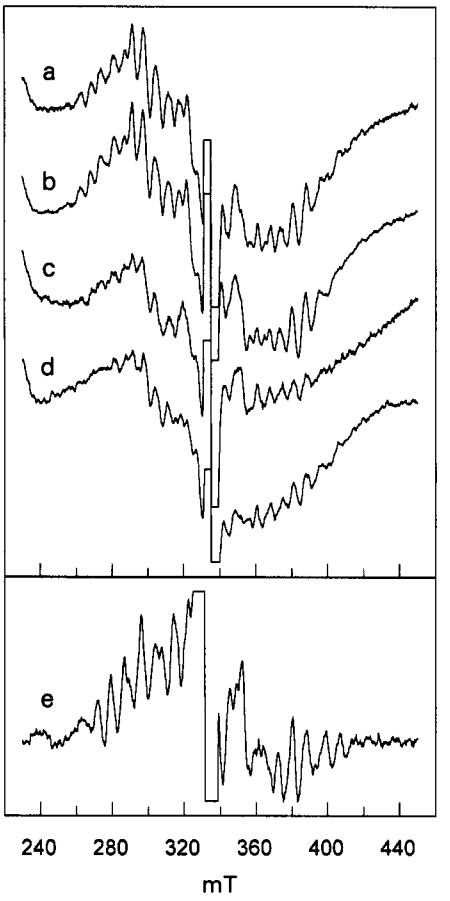

FIGURE 1: EPR spectra of dark-adapted, salt-washed/EGTA-treated PS-II membranes washed once in (a) 30 mM Cl^- and 5mM EGTA or three times in (b) 30 mM Cl^- and 50 μM EGTA, (c) 50 μM EGTA, or (d) 50 μM EGTA followed by one wash in 30 mM Cl^- and 50 μM EGTA. (e) Light minus dark spectrum of the sample from spectrum d after addition of PPBQ followed by illumination at 200 K. Instrument settings: 9.44 GHz; modulation amplitude, 2.2 mT; temperature, 10 K. Spectra a–d were measured at a microwave power of 20 mW, and spectrum e, at 31 mW.

RESULTS

Effects of Cl^- Depletion in Salt-Washed/EGTA-Treated PS-II: The S_2 State. The effects of depleting Cl^- and lowering the EGTA concentration in Ca^{2+}-depleted/EGTA-treated PS-II membranes were investigated. Figure 1a–d shows EPR spectra of dark-adapted PS-II membranes that were salt-washed, EGTA-treated, and subjected to a range of different washing procedures as described below.

Following a wash in 5 mM EGTA and 30 mM Cl^-, a dark-stable S_2 modified multiline signal was present (Figure 1a) as characterized by Boussac et al. (1989). Since the spectra in Figure 1a–d were recorded in dark-adapted samples, baseline signals (e.g., cyt *b*559, the Rieske center) underly the Mn multiline signal. In these membranes, no additional multiline signal could be generated by illumination at 200 K (not shown), indicating that most of the centers were modified after the treatment. Lowering the EGTA concentration to 50 μM in these membranes in the presence of Cl^- (30 mM) did not affect the dark-stable modified multiline signal (Figure 1b). However, when the EGTA concentration was lowered in the absence of Cl^-, only a residual modified multiline signal (~25%) was present (Figure 1c). It is possible that upon Cl^- depletion the S_2 state is not detected by EPR, as was observed following Cl^- depletion in functional PS-II (Ono et al., 1986; Boussac et al., 1992). Another possibility is that the Cl^--depleted S_2 state is not stable in the dark. To investigate the origin of the decrease in the multiline signal, the Cl^--free washed PS-II membranes were Cl^--reconstituted with 30 mM Cl^- in the presence of 50 μM EGTA and dark-adapted. The Cl^--reconstituted membranes showed the same residual amount of dark-stable modified multiline signal (Figure 1d) as the Cl^--free washed membranes (Figure 1c). In addition, a normal multiline signal was formed after illumination at 200 K (Figure 1e). This indicates that most of the centers lost the chelator-induced modification during the Cl^--free washes at a low concentration of EGTA, and that the centers were in S_1 following Cl^- reconstitution and dark adaptation. A small amount of S_3 signal was generated by the illumination at 200 K due to turnover from S_2 to S_3 in a small fraction of centers present in a dark-stable S_2 state as indicated by the residual modified multiline signal in the dark spectrum. The light-induced signal around 354 mT (g = 1.90) corresponds to Q_A^-–Fe^{2+}.

As expected, a normal S_2 multiline signal similar to that in Figure 1e also was observed after Ca^{2+} reconstitution, with 5 mM Ca^{2+}, of salt-washed/EGTA-treated PS-II with 30 mM Cl^- present (not shown). The possibility that the observed loss of the EGTA-induced modification of S_2 after Cl^--free washes followed by Cl^- reconstitution (Figure 1e) was due to contamination with Ca^{2+} can be ruled out for the following reasons: (1) There was very little variability from experiment

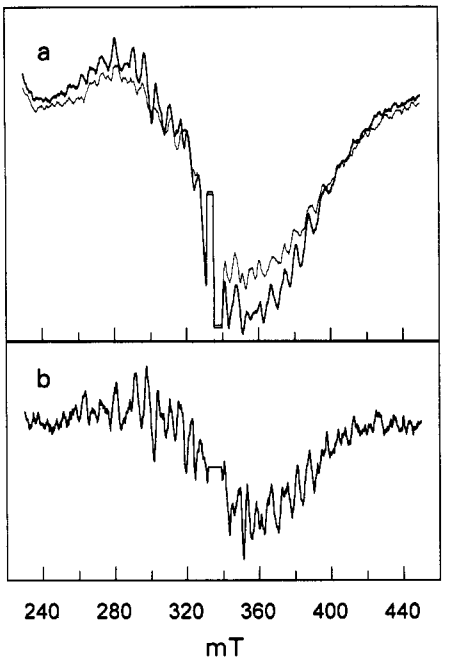

FIGURE 2: EPR spectra of salt-washed/EGTA-treated PS-II membranes, washed afterwards three times with 5 mM EGTA. (a) Dark spectra before (thin) and after (thick) addition of chloride in the dark, followed by 30 min of incubation in darkness on ice. (b) Chloride-induced spectrum from (a) (thick minus thin). The intensity of spectrum b was multiplied by 2 in comparison to spectrum a. Instrument settings were as in Figure 1; microwave power, 20 mW.

to experiment in the extent of the effect of Cl^- depletion on the EPR signals. (2) The presence of 50 μM EGTA during the experiment is far beyond the threshold for Ca^{2+} contamination, and residual amounts of contaminating Ca^{2+} were therefore trapped by EGTA. (3) Following the different treatments, the membranes, including the Cl^--reconstituted membranes described above, were equally inhibited in terms of oxygen evolution and showed the same extent of reconstituted oxygen-evolving activity when 6 mM Ca^{2+} was present. Thus, the observation that the EGTA-induced modification in the salt-washed/EGTA-treated membranes was reversed by Cl^- depletion in the presence of a low concentration of EGTA (50 μM) and in the absence of Ca^{2+} (Figure 1) strongly indicates that the modification of S_2 is due to binding of the chelator to PS-II. This conclusion and further conclusions from this work (see below) are summarized in Figure 5.

In a further investigation of the relationship between the chelator-induced modification of S_2 and Cl^- depletion, salt-washed/EGTA-treated PS-II membranes were washed in a Cl^--free solution in the presence of a high EGTA concentration (5 mM). Following this washing procedure, a small amount of dark-stable modified multiline signal was present (Figure 2a, thin line) with an intensity of approximately 15% compared to that of a control sample washed in 5 mM EGTA and 30 mM Cl^- (see, e.g., Figure 1a). Addition of Cl^- to this sample in complete darkness resulted in an increased intensity of the dark-stable modified multiline signal (Figure 2a, thick line). The intensity of the Cl^--induced multiline signal (Figure 2b) was about 50% of that of the control (Figure 1a).

Figure 1 showed that the EGTA-induced modification in salt-washed/EGTA-treated PS-II was reversed by lowering the chelator concentration in the absence of Cl^-. However, Figure 2 shows that this effect was largely overruled when the chelator concentration was kept at 5 mM. Nevertheless, the chelator-modified S_2, which is stable in the dark, was not detected by EPR after Cl^- depletion, indicating that the Cl^--depletion treatment prevented the detection of the chelator modification. Thus, Cl^- depletion modified the S_2 state to a state which is not detected by EPR. Moreover, provided that the EGTA concentration was sufficiently high (5 mM), the EGTA- and Cl^--depletion treatments that modify S_2 in Ca^{2+}-depleted PS-II were additive (see Figure 5).

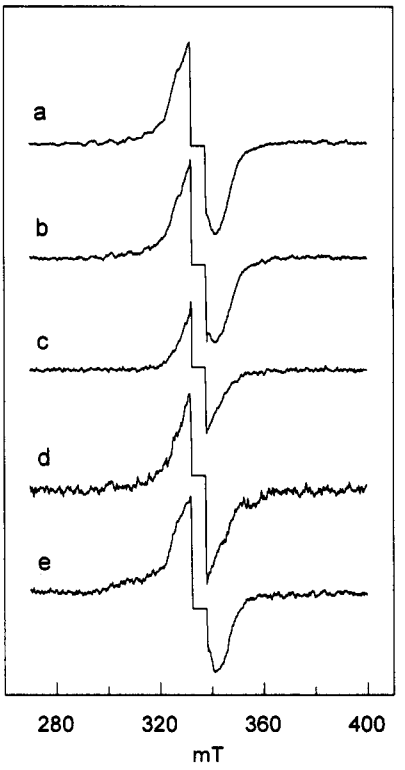

FIGURE 3: Light minus dark spectra of salt-washed/EGTA-treated PS-II membranes that were illuminated for 3 min at 0 °C in the presence of PPBQ and rapidly frozen afterwards. The membranes were washed once in (a) 30 mM Cl^- and 5 mM EGTA or three times in (b) 30 mM Cl^- and 50 μM EGTA, (c) 50 μM EGTA, (d) 5 mM EGTA, or (e) 50 μM EGTA, followed by one wash in 30 mM Cl^- and 50 μM EGTA. Spectra were recorded under the chosen instrument settings, which were as in Figure 1 except that the modulation amplitude was 0.22 mT and the microwave power was 2 mW. No (negative) contribution from the S_2 multiline signal intensity is present in the difference spectra.

The state that is present in salt-washed/EGTA-treated PS-II after Cl^- depletion in the presence of a low EGTA concentration (see Figure 1c), resulting in the loss of the EGTA-induced modification, was further investigated by rapid addition of Cl^- in darkness following dark-adaptation. The results were not very reproducible in that the addition of Cl^- resulted in the appearance of variable amounts of normal multiline signal (not shown). This problem probably originates from the decay of the normal S_2 state that is formed during Cl^- addition and mixing. Nevertheless, these results indicate that the S_2 state after Cl^- depletion in Ca^{2+}-depleted PS-II does not exhibit a multiline signal and is probably more stable in the dark than the normal S_2 state (Figure 5).

Effects of Cl^- Depletion in Salt-Washed/EGTA-Treated PS-II: The S_3 State. Figure 3 shows light-induced EPR signals from salt-washed/EGTA-treated membranes following continuous illumination at 0 °C. In membranes washed in 30 mM Cl^- and 5 mM EGTA a light-induced signal around $g = 2$ is observed with a peak to trough width of about 13 mT (Figure 3a). This signal is observed in Ca^{2+}-depleted membranes lacking the extrinsic 17- and 23-kDa polypeptides and corresponds to the formal S_3 state (Boussac et al. 1990a). Lowering the EGTA concentration to 50 μM in these

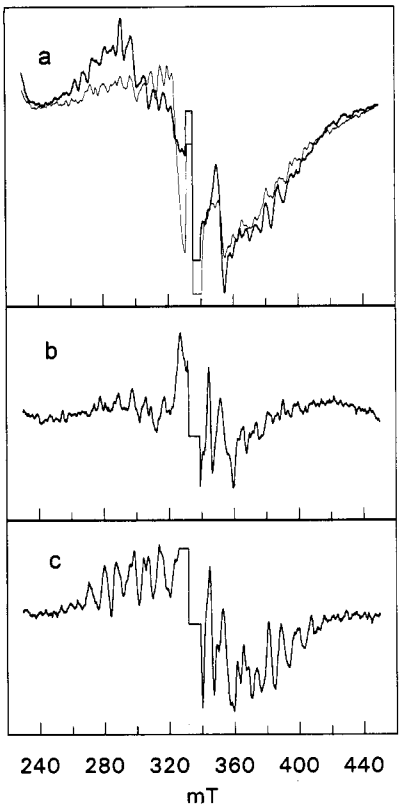

FIGURE 4: Dark spectra of salt-washed PS-II. Salt washing was followed by three washes in Ca^{2+}-free solutions containing 30 mM Cl^- and (a) (thin) 5 mM MES (pH 6.5) or (thick) 500 mM MES (pH 6.5). Spectra b and c are light minus dark spectra of the samples from (a) containing 500 and 5 mM MES, respectively, after addition of PPBQ followed by illumination at 200 K. Instrument settings were as in Figure 2.

membranes in the presence of Cl^- (30 mM) did not affect the properties of the S_3 signal (Figure 3b). However, after washes without Cl^- in 50 μM EGTA (Figure 3c) or 5 mM EGTA (Figure 3d), the light-induced S_3 signal was narrower (<10 mT) and unresolved due to the overlap with the stable tyrosine radical. The narrow S_3 signal is typically observed in regular PS-II membranes following Cl^- depletion (Baumgarten et al., 1991; Boussac et al, 1992). Thus, Cl^- depletion in Ca^{2+}-depleted PS-II not only modifies the S_2 state (see above) but also modifies the S_3 state, resulting in a narrower S_3 signal (see Figure 5). This modification was completely reversed by Cl^- reconstitution in 30 mM Cl^- and 50 μM EGTA, resulting in the 13 mT wide S_3 signal (Figure 3e).

A MES-Induced S_2 Dark-Stable Modified Multiline Signal. It was shown above that chelator binding is responsible for the modification of the S_2 multiline signal in Ca^{2+}-depleted PS-II. Nevertheless, the salt treatment without a chelator, followed by washes in Ca^{2+}-free buffer solutions, resulted in a small amount of dark-stable modified multiline signal. Since the Ca^{2+}-free solutions contained only Cl^- and MES as a pH buffer, we investigated the possibility that the modification was due to the presence of MES. This was done by washing the salt-treated membranes (without a chelator) in a Ca^{2+}-free solution containing 5, 25, or 500 mM MES and 30 mM Cl^-.

Following washes in 500 mM MES, a dark-stable modified multiline signal was observed (Figure 4a; thick line) similar to that observed using a chelator (see above). In this sample only a small amount of normal multiline signal was formed after illumination at 200 K (Figure 4b), indicating that most of the centers were present in a dark-stable S_2 state. In 5 mM

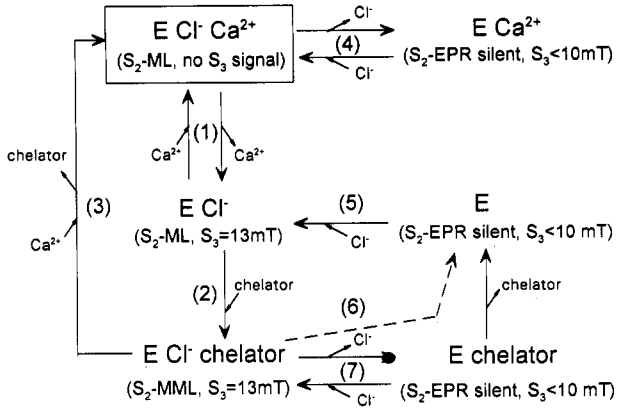

FIGURE 5: Schematic representation of the effects of Ca^{2+} and Cl^- depletion in the oxygen-evolving enzyme (E) on the EPR properties of S_2 and S_3 (see Results and Discussion). Functional PS-II (E Cl^- Ca^{2+}) is indicated in the box. ML = regular multiline signal. MML = chelator-modified multiline signal. Steps: (1) Ca^{2+} depletion by salt treatment in a low concentration (50 μM) of EGTA followed by Ca^{2+} reconstitution (Boussac & Rutherford, 1988a,b; Boussac et al., 1990a). (2) Modification of S_2 by the chelator in salt-washed PS-II (Boussac et al., 1990a; see also Figure 4). (3) Ca^{2+} reconstitution in salt-washed/EGTA-treated PS-II (Boussac et al., 1989). (4) Cl^- depletion in functional PS-II followed by Cl^- reconstitution (Ono et al., 1986; Boussac et al., 1992; Baumgarten et al., 1990). (6) Cl^- depletion in salt-washed/EGTA-treated PS-II in the presence of a low concentration (50 μM) of EGTA (Figures 1c and 3c) followed by (5) Cl^- reconstitution (Figures 1d,e and 3e). (7) Cl^- depletion in salt-washed/EGTA-treated PS-II in the presence of a high concentration (5 mM) of EGTA [Figures 2a (thin) and 3d] followed by Cl^- reconstitution [Figure 2a(thick),b].

MES, the modified multiline signal was nearly absent (Figure 4a, thin line) and a normal multiline signal was generated instead following 200 K illumination (Figure 4c), indicating that in 5 mM MES most of the centers were present in the S_1 state. In 25 mM MES an intermediate amount of dark-stable modified multiline signal was present (not shown). Control samples in which 500 mM NaCl was added to salt-washed PS-II already containing 25 mM MES showed no additional modified multiline signal.

Note that this particular preparation is contaminated with the Rieske iron–sulfur center in the reduced form as indicated by the dark-stable signal around 350 mT (g = 1.9) in Figure 4a. There is also contamination with PS-I manifested as the light-induced signals at 346 mT ($g \sim 1.95$) and 357 mT ($g \sim 1.89$) in Figure 4b,c corresponding to iron–sulfur centers which are electron acceptors in PS-I and are stably photoreduced after continuous illumination at 200 K.

The concentration of MES necessary for complete modification was much larger (hundreds of millimolar) than those of the chelators EGTA and EDTA (millimolar), indicating that the binding affinity of MES is much lower than those of the chelators.

DISCUSSION

The effects of the various Cl^- and Ca^{2+} depletion and reconstitution treatments in PS-II relevant to this work are shown schematically in Figure 5.

In this study, experimental conditions were obtained which reversed the chelator modification in salt-washed/EGTA-treated PS-II, without Ca^{2+} reconstitution. Thus, it seems very unlikely that the modification of S_2, resulting in a dark-stable modified S_2 multiline signal, is due to removal of Ca^{2+}, and the results therefore indicate that the modification is due to binding of the chelator to PS-II. Chelator binding to PS-II occurs upon release of Ca^{2+}, and in addition, both events occur

most easily in S_3 (Boussac & Rutherford, 1988b; Boussac et al., 1985, 1990a). Upon Ca^{2+} reconstitution, the modification of S_2 is eliminated (Boussac et al., 1989), and it is shown above that this is likely to be due to dissociation of the chelator from PS-II. From these observations it seems clear that the access of the chelators to PS-II is greatly enhanced in S_3 when Ca^{2+} is released from the functional site. This may have some relevance to the role(s) of Ca^{2+} in functional oxygen-evolving PS-II [discussed in Rutherford et al. (1992)]. It has been suggested that Ca^{2+} may play a role in controlling substrate access to the active site of the oxygen-evolving enzyme [reviewed in Rutherford et al. (1992)].

Components that modify S_2, resulting in a modified multiline signal, include citrate, EGTA, EDTA [Boussac et al., 1989, 1990a; see also Sivaraja and Dismukes (1989) and Ono and Inoue (1990)], pyrophosphate (Zimmermann et al., 1993), and MES (this work). The questions which arise are (1) Where do the components that modify S_2 bind? and (2) How do they interact with the manganese cluster? That the components mentioned above modify S_2 in the same way, resulting in a characteristic dark-stable modified multiline signal, indicates that a specific binding site is involved. Observations using ESEEM suggested that the chelator EGTA was close the manganese cluster (Zimmermann et al., 1993). A common chemical feature of the components that modify S_2 is the presence of anionic groups containing oxygen—carboxylic acid (EGTA, EDTA, citrate), pyrophosphate, and sulfonic acid (MES)—indicating that modification of the manganese cluster includes an interaction with the anionic oxygens. Further information about the characteristics of chelator binding in Ca^{2+}-depleted PS-II comes from the observation that treatment with phthalic or terephthalic acid (10 mM), at pH 6.5 in the presence of Cl^- (30 mM), does not result in the generation of a modified stable multiline signal (Boussac et al., 1990a). This may indicate that chelator binding requires an interaction with polyanionic groups of a given configuration that is obtained with flexible molecules [see Boussac et al. (1990)]. This may further suggest that the modification of S_2 by MES, which contains a single sulfonic acid group per molecule, occurs upon binding of more than one molecule per PS-II.

The key factor in the experiment that reversed the chelator-induced modification in the absence of Ca^{2+} was the removal of Cl^- in the presence of a low concentration of EGTA (50 μM). Thus, Cl^- appears to support the binding of the chelator to PS-II, and Cl^- depletion lowers significantly the binding affinity of the chelator. This phenomenon is quite surprising since the anionic chelators may have been expected to compete with Cl^- for a common binding site. Many inhibitors of oxygen evolution (e.g., OH^-, NH_3, amines, SO_4^{2-}, F^-) seem to be competitive with Cl^- binding [reviewed by Rutherford et al. (1992)]. Earlier attempts made in our laboratory to reverse the effect of the chelator in increasing concentrations of Cl^- in the absence of Ca^{2+} were unsuccessful (G. N. Johnson and A. W. Rutherford, unpublished). Yachandra et al. (1993) have suggested that Cl^- is a ligand to the Mn cluster. It can be speculated that ligation of Cl^- to the manganese cluster induces an enhanced affinity of the anionic groups of the chelator to the manganese cluster, e.g., due to a changed redox distribution between the Mn ions. It is also possible that chelator binding is stabilized by protonation events occurring in parallel to Cl^- reconstition. The pH dependence of Cl^--reconstituted oxygen evolution in PS-II indicated that one protonation event occurs per reconstituted Cl^- (Homann, 1988b). The observed Cl^--stimulated chelator binding may have some relevance to the role(s) of Cl^- in the mechanism of O_2 evolution, in that Cl^- controls functional association of the substrate to PS-II. Several suggestions on the role(s) of Cl^-, found in the literature, are summarized in Rutherford et al. (1992).

It was shown in the Results that Cl^- depletion of Ca^{2+}-depleted/EGTA-treated PS-II modified the EPR spectroscopic properties of both the S_2 and the S_3 state. The chelator-modified S_2 state, present when Cl^- depletion is done in a high concentration of EGTA (5 mM), is not detected by EPR, and the modified S_3 signal is narrow, having a width < 10 mT. These modifications were completely reversed by reconstitution with Cl^-, resulting in the appearance of the chelator-modified S_2 multiline signal and a 13 mT wide S_3 signal (Figure 3e). The Cl^--dependent behavior of S_2 that was observed in Ca^{2+}-depleted PS-II is similar to that following Cl^- depletion in functional PS-II (Ono et al., 1986; Boussac & Rutherford, 1994). In addition, the type of S_3 signal that was observed in Ca^{2+}-depleted, Cl^--depleted PS-II is similar to that observed following Cl^- depletion in functional PS-II after treatment with SO_4^{2-} (Boussac et al., 1992) or F^- (Baumgarten et al., 1990), also having a width smaller than 10 mT. Thus, the effects of Cl^- depletion on the EPR properties of S_2 and S_3 in Ca^{2+}-depleted PS-II appear to be similar to those observed following Cl^- depletion in regular PS-II membranes in which the functional Ca^{2+} is thought to be present: the formation of an S_2 state not detected by EPR and a narrower S_3-signal (<10 mT). These results indicate that the Cl^--related EPR properties of S_2 and S_3 described above are Ca^{2+}-independent.

On the basis of an enzymological study in PS-II depleted of Cl^- and Ca^{2+}, Waggoner and Yocum (1990) concluded that activation of oxygen evolution in PS-II requires binding of Cl^- after binding of Ca^{2+}. This is in apparent contradiction with our conclusion based on the EPR phenomena described above. The conclusions may be reconciled by suggesting that different Cl^--binding sites are involved, which would imply that the Cl^--binding site in Ca^{2+}-depleted PS-II probed by EPR is not related to oxygen-evolving activity. The very different experimental conditions used in the two studies could make direct comparisons of the EPR and enzymological studies unreliable. Nevertheless, the data points of Waggoner and Yocum (1990), although clearly showing a requirement of both Ca^{2+} and Cl^- for oxygen-evolving activity, are not as clear in indicating an ordered binding requirement. A more precise enzymological study is required to resolve this point. Thus that Cl^-, essential in the charge accumulation cycle, remains bound when Ca^{2+} is removed, is the most straightforward explanation and remains the most favored interpretation of our data.

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