# Properties of the Chloride-Depleted Oxygen-Evolving Complex of Photosystem II Studied by Electron Paramagnetic Resonance

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ABSTRACT: The effects of different $Cl^-$ depletion treatments in photosystem II (PS-II)-enriched membranes have been investigated by electron paramagnetic resonance (EPR) spectroscopy and by measurements of oxygen-evolving activity. The results indicated that the oxygen-evolving complex of PS-II exhibits two distinct $Cl^-$-dependent properties. (1) After $Cl^-$-free washes at pH 6.3, a reversibly altered distribution of structural states of PS-II was observed, manifested as the appearance of a $g = 4$ EPR signal from the $S_2$ state in a significant fraction of centers (20−40%) at the expense of the $S_2$ multiline signal. In addition, small but significant changes in the shape of the $S_2$ multiline EPR signal were observed. Reconstitution of $Cl^-$ to $Cl^-$-free washed PS-II rapidly reversed the observed effects of the $Cl^-$-free washing. The anions, $SO_4^{2-}$ and $F^-$, which are often used during $Cl^-$ depletion treatments, had no effect on the $S_2$ EPR properties of PS-II under these conditions in the absence or presence of $Cl^-$. Flash experiments and measurements of oxygen evolution versus light intensity indicated that the two structural states observed after the removal of $Cl^-$ at pH 6.3 originated from oxygen-evolving centers exhibiting a lowered quantum yield of water oxidation. (2) Depletion of $Cl^-$ in PS-II by pH 10 treatment reversibly inhibited the oxygen-evolving activity to ≈15%. The pH 10 treatment depleted the $Cl^-$ from a site which is considered to be equivalent to that studied in most earlier work on $Cl^-$-depleted PS-II. The $S_2$ state in pH 10/$Cl^-$-depleted PS-II was reversibly modified to a state from which no $S_2$ multiline EPR signal was generated and which exhibited an intense $S_2$ $g = 4$ EPR signal corresponding to at least 40% of the centers but possibly to a much larger fraction of centers. The state responsible for the intense $S_2$ $g = 4$ signal generated under these conditions is unlike that observed after removal of $Cl^-$ from PS-II at pH 6.3, in that this state was more stable in the dark, showing a half-decay time of ≈1.5 h at 0 °C, and was unable to undergo further charge accumulation. Nevertheless, a fraction of centers, probably different from those exhibiting the $S_2$ $g = 4$ signal, was able to advance to the formal $S_3$ state, giving rise to a narrow EPR signal around $g = 2$. Addition of the anions $SO_4^{2-}$ or $F^-$ to pH 10/$Cl^-$-depleted PS-II affected the properties of PS-II, resulting in EPR properties of the $S_2$ state similar to those reported earlier following $Cl^-$ depletion treatment of PS-II in the presence of these anions. Surprisingly, after addition of $F^-$, the $g = 4$ EPR signal showed a damped flash-dependent oscillation. In addition, a narrow signal around $g = 2$, corresponding to the formal $S_3$ state, also showed a damped flash-dependent oscillation pattern. The presence of oscillating EPR signals (albeit damped) in $F^-$-treated pH 10/$Cl^-$-depleted PS-II indicates functional enzyme turnover. This was confirmed by measurements of the oxygen-evolving activity versus light intensity which indicated that in ∼45% of oxygen-evolving centers the enzyme turnover was slowed by a factor of 2. The distinct $Cl^-$ depletion effects in PS-II observed under the two different $Cl^-$ depletion treatments are considered to reflect the presence of two distinct $Cl^-$-binding sites in PS-II.

Photosynthetic water oxidation, resulting in the formation of molecular oxygen and proton release, is thought to occur upon photoaccumulation of four positive charges in an enzyme 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). A cluster of four manganese ions, which is thought to be present at the lumenal side of the membrane-spanning photosystem II protein complex (PS-II),[1] plays a central role in the charge accumulation cycle. The kinetic properties of the Mn oxidation states under different experimental conditions have been characterized in detail [reviewed in Rutherford et al. (1992) and Debus (1992)]. In addition to the Mn cluster, the ions $Ca^{2+}$and $Cl^-$ are essential for oxygen-evolving activity. Three extrinsic polypeptides 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-evolving activity [reviewed by Murata and Miyao (1985)]. The 33 kDa polypeptide stabilizes the Mn cluster. The 17 and 23 kDa polypeptides play a role in retention of functional $Ca^{2+}$ and $Cl^-$ (Murata & Miyao, 1985; Homann, 1988a). In the study of the roles

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[1] Abbreviations: PS-II, the photosystem II protein complex; TyrD, side path electron donor of PS-II responsible for EPR signal $II_{slow}$; $Q_A$ and $Q_B$, primary and secondary quinone electron acceptors of PS-II; CW, continuous wave; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone.

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of $Ca^{2+}$ and $Cl^-$ in the charge accumulation cycle, a number of methods have been developed to specifically deplete these anions from PS-II, resulting in reversible inhibition of oxygen evolution while the Mn cluster is retained in its site (Homann, 1987; Debus, 1992). Depletion of $Ca^{2+}$ involves dissociation of the 17 and 23 kDa proteins by washing of the PS-II membranes in a high concentration of NaCl (Ghanotakis et al., 1984b) or treatment at a low pH (Ono & Inoue, 1988). Methods for depletion of $Cl^-$ from PS-II include a short treatment at a high pH (pH 10) or treatments at slightly elevated pH (pH 7.5) in the presence of the inhibitory counteranions $SO_4^{2-}$ or $F^-$ (Homann, 1987). Several lines of evidence indicate that depletion of functional $Ca^{2+}$ or $Cl^-$ from PS-II results in inhibition of the $S_3$ to $S_0$ transition of the charge accumulation cycle [reviewed in Rutherford et al. (1992) and Boussac and Rutherford (1994a)].

Several suggestions for the roles of $Ca^{2+}$ and $Cl^-$ in the mechanism of photosynthetic water oxidation have been made [reviewed in Debus (1992) and Rutherford et al. (1992)], including the possibility that these anions may regulate protonation/deprotonation events (Boussac & Rutherford, 1988a; Homann, 1988b; Boussac et al., 1992; Lübbers et al., 1993). Furthermore, it has been suggested that these anions play a role in controlling the access and binding of the substrate [reviewed in Rutherford et al. (1992); see also van Vliet et al. (1994)]. With respect to the role of $Cl^-$, the latter suggestion was partly based on observations that $Cl^-$ protects against reductive attack by a range of substrate-like inhibitors [see Sandusky and Yocum (1984, 1986) and Mei and Yocum (1993)]. The extrinsic polypeptides themselves also protect against reductive attack (Ghanotakis et al., 1984a; Mei & Yocum, 1993). Since $Cl^-$ seems to enhance the association of the 17 and 23 kDa extrinsic polypeptides to PS-II (Homann, 1988a,c), some of the protective effects of $Cl^-$ may result from its stabilizing effect on protein associations.

Many studies on the magnetic properties of the oxygen-evolving complex have been done using EPR spectroscopy. In untreated PS-II, no signals from states other than the $S_2$ state have been detected by conventional CW EPR. However, after $Ca^{2+}$ depletion, an EPR signal around $g = 2$ with a width of 16.4 mT was observed, corresponding to the formal $S_3$ state [Boussac et al., 1989; see also Sivaraja et al. (1989)]. Boussac et al. (1989, 1990) have proposed that, upon the $S_2$ to $S_3$ transition in $Ca^{2+}$-depleted PS-II, the oxidation state of the Mn cluster remains unchanged and that an organic species is oxidized instead giving rise to an $S = {}^1/_2$ radical magnetically interacting with the $S = {}^1/_2$ Mn cluster. The radical species was proposed to be an oxidized histidine on the basis of its absorption spectrum in the ultraviolet (Boussac et al., 1990). Although this assignment has received further support from a study by FTIR difference spectroscopy (Berthomieu & Boussac, 1995), it is not definitive [discussed in Debus (1992) and Rutherford and Boussac (1992)]. Recently, Gilchrist et al. (1995) have proposed, on the basis of an ESE-ENDOR investigation in $Ca^{2+}$-depleted PS-II, that the radical signal originates from oxidized TyrZ, the electron transfer intermediate between the Mn cluster and the primary electron donor $P_{680}$. Besides their formation in $Ca^{2+}$-depleted PS-II, $S_3$ signals also were observed after inhibition of oxygen evolution by treatments in the presence of $F^-$ (Baumgarten et al., 1990), $SO_4^{2-}$ (Boussac et al., 1992), $NH_3$ (Andréasson & Lindberg, 1992; Hallahan et al., 1992), or acetate (McLachlan et al., 1993) which are thought to displace the functional $Cl^-$ in PS-II.

In untreated PS-II, the EPR spectrum of the $S_2$ state is dominated by a characteristic multiline EPR signal at $g = 2$ (Dismukes & Siderer, 1981). This signal can be generated by illumination treatments allowing for a single stable charge separation, e.g. illumination with a single flash at room temperature (Dismukes & Siderer, 1981), or with continuous illumination at 200 K (Brudvig et al., 1983). The $S_2$ multiline signal is attributed to a ground state spin $S = {}^1/_2$ probably arising from a mixed valence Mn tetramer [see Britt et al. (1992) and references therein]. Depending on the conditions, the $S_2$ state also exhibits a signal around $g = 4$ [reviewed in Rutherford et al. (1992)]. This signal is less well-characterized than the $S_2$ multiline signal but is thought to arise from a spin $S = {}^3/_2$ or $S = {}^5/_2$ ground or excited state of the mixed valence Mn cluster [see Astashkin et al. (1994) and Smith et al. (1993)]. The nature of the $S_2$ $g = 4$ signal seems to depend on the pretreatment of the enzyme (Smith & Pace, 1995). The $S_2$ $g = 4$ and $S_2$ multiline signals probably originate from two different structural states of the oxygen-evolving complex with different magnetic properties of the Mn cluster (dePaula et al., 1985; Zimmermann & Rutherford, 1986; Hansson et al., 1987).

The multiline EPR signal from $S_2$ seems to be related to functional binding of $Cl^-$ to PS-II as indicated by the loss of the ability to generate the $S_2$ multiline signal following inhibition of oxygen evolution by $Cl^-$ depletion in the presence of $SO_4^{2-}$ (Ono et al., 1986, 1987; Boussac et al., 1992; Boussac & Rutherford, 1994b) or $F^-$ (Damoder et al., 1986; Baumgarten et al., 1990; DeRose et al., 1995). After inhibition of oxygen evolution by $Cl^-$ depletion in the presence of $SO_4^{2-}$, a modified $S_2$ state which was not detected by EPR was generated and was converted to the normal $S_2$ state by rapid addition of $Cl^-$ in darkness, resulting in the reconstitution of the $S_2$ multiline EPR signal (Ono et al., 1986). A similar $Cl^-$ depletion effect on the EPR properties of $S_2$ was observed in $Ca^{2+}$-depleted PS-II, indicating that this $Cl^-$ depletion effect was independent of $Ca^{2+}$ (van Vliet et al., 1994).

The relationship between the appearance of the $S_2$ $g = 4$ EPR signal from the Mn cluster and the functional and biochemical properties of the oxygen-evolving complex is poorly understood [discussed in Rutherford et al. (1992)]. The $S_2$ $g = 4$ signal, observed in untreated PS-II resuspended in sucrose buffer, seems to be suppressed in favor of the $S_2$ multiline signal by the presence of glycerol, ethylene glycol, and ethanol which are used as cryoprotectants or solvents (Zimmermann & Rutherford, 1986a). However, these effects seem to vary depending on the experimental conditions [see Beck and Brudvig (1988) or Casey and Sauer (1984)]. The $S_2$ $g = 4$ signal in untreated PS-II was shown to originate from the functional charge accumulation cycle (Zimmermann & Rutherford, 1986a; Andréasson, 1990). Even so, it has also been suggested that the appearance of the $S_2$ $g = 4$ signal is related to inhibition of oxygen evolution due to the release of $Cl^-$ (Beck & Brudvig, 1988) on the basis of the observation that the $S_2$ $g = 4$ signal was enhanced after treatment of PS-II with $F^-$ [e.g. Casey and Sauer (1984)] or $NH_3$ [e.g. Beck and Brudvig (1986)] which were thought to displace the functional $Cl^-$. However, the $S_2$ $g = 4$ signal intensities generated following displacement of the functional $Cl^-$ in PS-II by various counteranions did not correlate to

the extent of inhibited oxygen-evolving activity (Ono et al., 1987).

In this report, the properties of $Cl^-$-depleted PS-II were investigated under various experimental conditions, with the aim of relating the biochemical status of $Cl^-$-depleted PS-II to the observed EPR properties.

## MATERIALS AND METHODS

PS-II-enriched membranes were prepared according to the method of Berthold et al. (1981) with the modifications of Ford and Evans (1983). The oxygen-evolving activity of these membranes was ≈500 $\mu$mol of $O_2$ per milligram of chlorophyll per hour. Prior to use for further treatments (see below), the PS-II membranes were stored at −80 °C in a buffer solution containing 25 mM MES (pH 6.5), 0.3 M sucrose, and 10 mM NaCl.

$Cl^-$ depletion in PS-II was done by a short treatment at pH 10 (see below) as described by Homann (1993). The principle of the $Cl^-$ depletion treatment is based on the idea that the 17 and 23 kDa extrinsic polypeptides are involved in retention of $Cl^-$ in the functional site (Homann, 1985, 1988a). The short treatment at pH 10 is thought to induce a transient dissociation of the 17 and 23 kDa extrinsic polypeptides, resulting in the release of $Cl^-$ from its site (Homann, 1985, 1988a). Prior to the pH 10 treatment, the $Cl^-$ concentration in untreated PS-II membranes was lowered by three washes (resuspension, dilution, and centrifugation) in a $Cl^-$-free buffer solution containing 5 mM MES (pH 6.3) and 0.5 M sucrose.

In some experiments, where indicated, PS-II membranes were washed in a $Cl^-$-free buffer solution as described above except that the pH was adjusted to pH 6.5. No differences between the effects of the washes at pH 6.3 or the washes at pH 6.5 were observed. The PS-II membranes that were repetitively washed in $Cl^-$-free buffer solutions at pH 6.3 or pH 6.5 will be referred to as $Cl^-$-free-washed PS-II.

Following resuspension and dilution of the $Cl^-$-free-washed PS-II membranes to a chlorophyll concentration of 125 $\mu$g/mL in a buffer-free solution containing 0.4 M sucrose, the pH was increased to pH 10 by addition of 15 mM (15 $\mu$L/mL of 1.0 M) CAPS (pH 10). After 10−35 s of incubation at pH 10, the pH was lowered to pH 7.3 by addition of 45 mM (45 $\mu$L/mL of 1.0 M) HEPES (pH 7.3), which was, unless stated otherwise, directly followed by lowering the pH to pH 6.3 by addition of 45 mM (45 $\mu$L/mL of 1.0 M) of unneutralized MES followed by 10 min of incubation. Addition of anions (as their sodium salt) to pH 10/$Cl^-$-depleted PS-II was done at pH 7.3, i.e. under conditions in which irreversible inhibition of oxygen evolution is minimized and yet PS-II is still sensitive to treatments that affect $Cl^-$-dependent oxygen-evolving activity (Rashid & Homann, 1992; Homann, 1993). Following 10−20 min of incubation, the pH was lowered to pH 6.3 as described above. The pH 10 treatment and addition of anions were done while stirring at 4 °C under dim light. To minimize possible $Cl^-$ contamination, the experiments were done using sucrose BDH ARISTAR (<0.5 ppm $Cl^-$).

Following the treatments described above, the content of the 17, 23, and 33 kDa extrinsic polypeptides in the PS-II membrane preparations was determined by sodium dodecyl sulfate (SDS) gel electrophoresis and subsequent Western blotting. SDS gel electrophoresis was carried out as described by Laemmli (1970), except that 750 mM instead of 375 mM Tris (pH 8.8) was present in the resolving gel, and 6 M urea was added to both gel and sample buffer. Western blotting was carried out as described by Seidler (1994), except that the tank blot device used for protein transfer onto the poly(vinylidene fluoride) (PVDF) membrane was from Biorad. Furthermore, the PVDF membrane was simultaneously incubated with the antibodies against the three extrinsic polypeptides. The antibodies were kindly provided by Dr. C. Jansson, Stockholm.

The membranes were resuspended at 2.5−15 mg of chlorophyll/mL, put in calibrated quartz EPR tubes, dark adapted, frozen in the dark, and stored in liquid nitrogen until used for EPR measurements. Further additions to these membranes were done in the EPR tube in the dark after thawing. Where indicated, illumination of the samples was done following addition in darkness of the external electron acceptor PPBQ dissolved in dimethyl sulfoxide.

Continuous illumination of the samples was done, using an 800 W projector through 2 cm water and an infrared filter, in a nonsilvered Dewar flask containing ethanol cooled to 198 K with solid $CO_2$ or cooled to 0 °C with liquid nitrogen. Flash illumination at room temperature was provided by an Nd-Yag laser (15 ns, 300 mJ, 532 nM).

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

Measurements of oxygen-evolving activity were done using a Clark-type electrode, at 25 °C under continuous light. The measurements were done under near-saturating light at a chlorophyll concentration of 20 $\mu$g/mL or under nonsaturating light at a chlorophyll concentration of 40 $\mu$g/mL, and 0.5 mM PPBQ was added as an external electron acceptor. The light intensity was varied using calibrated neutral gray (Balzers) filters.

## RESULTS

*Effects of $Cl^-$-Free Washes at pH 6.5.* After $Cl^-$-free washes at pH 6.5, prior to pH 10/$Cl^-$ depletion treatment, the oxygen-evolving activity of the PS-II membranes measured in the presence of $Cl^-$ (10 mM) was about 85% relative to that in untreated PS-II, indicating a fraction (15%) of irreversible inhibition after the $Cl^-$-free washes. This was probably due to Mn release from the functional site of PS-II as indicated by the apearance of a small six-line signal around $g = 2$ in the EPR spectrum originating from hexaquomanganese(II) (not shown).

In the absence of $Cl^-$, the oxygen-evolving activity of $Cl^-$-free-washed PS-II membranes was only slightly inhibited (activity ≈ 80% of that after reconstitution of $Cl^-$). The apparent $Cl^-$ affinity for reconstitution of oxygen evolution from 80 to 100% in $Cl^-$-free-washed PS-II was about 0.4 mM (not shown). The level of inhibition of oxygen evolution was dependent on the light intensity and increased at lower light intensities (Figure 1). The oxygen-evolving activity in $Cl^-$-free-washed PS-II, when extrapolated to full light saturation, was similar to that after reconstitution of $Cl^-$ (10 mM) (Figure 1), indicating that, apart from the fraction (15%) of irreversibly inhibited oxygen evolution, the removal of $Cl^-$ did not affect the number of oxygen-evolving centers but rather influenced the enzyme kinetics. Since the EPR studies shown below indicate specific $Cl^-$-

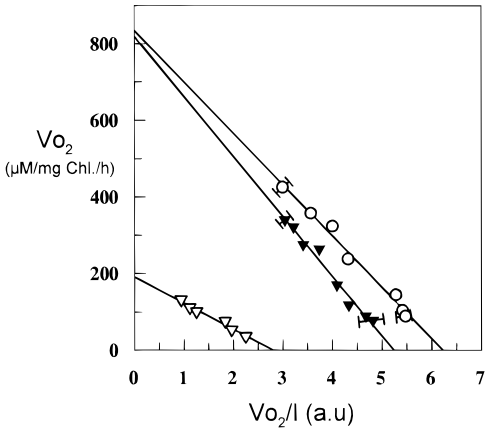


FIGURE 1: Plot of oxygen evolution ($V_{O_2}$) versus oxygen evolution over light intensity ($V_{O_2}/I$). The measurements were done either after three washes of PS-II membranes in a $Cl^-$-free buffer solution contaning 5 mM MES (pH 6.3) and 0.5 M sucrose and resuspension in the same buffer (▼ and ○) or after subsequent pH 10/$Cl^-$ depletion treatment following the $Cl^-$-free washes and followed by reconstitution with $F^-$ (25 mM) and resuspension in 10 mM MES (pH 6.3), 0.5 M sucrose, and 25 mM $F^-$ (▽). The measurements were done in a buffer solution containing 10 mM MES (pH 6.3) and 0.5 M sucrose in the absence (▼ and ▽) or presence of 10 mM $Cl^-$ (○). Each data point is the average of at least six measurements. The error in the data points from the $F^-$-reconstituted PS-II membranes matched or was smaller than the symbol size.

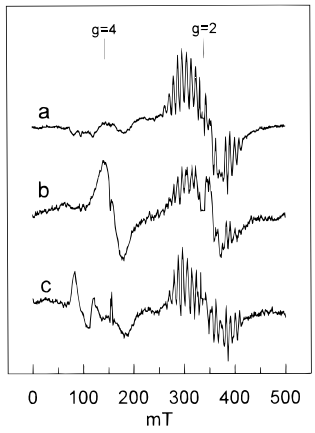


FIGURE 2: Light minus dark EPR spectra of PS-II membranes that were illuminated for 3 min at 200 K in the presence of PPBQ (1 mM). The PS-II membranes were (a) washed three times in 5 mM MES (pH 6.5) and 10 mM $Cl^-$ and resuspended in the same buffer solution or (b) washed twice in 5 mM MES (pH 6.5) and 0.1 M sucrose, followed by two washes and resuspension in 5 mM MES (pH 6.5) (i.e. in the absence of sucrose). (c) The $Cl^-$-free-washed sample used for spectrum b was thawed, and $Cl^-$ (50 mM) was added rapidly (30 s) in darkness and refrozen: instrument settings, 9.42 GHz; modulation amplitude, 2.2 mT; temperature, 10 K; and microwave power, 31 mW.

dependent modifications at the electron donor side of PS-II while no $Cl^-$-dependent effects were observed at the electron acceptor side, it seems most likely that the observed changes in the enzyme kinetics are related to donor side phenomena.

In control samples which were washed in the presence of $Cl^-$ (10 mM) without sucrose (see below), the $S_2$ state exhibited a characteristic multiline EPR signal and no $g = 4$ signal was observed (Figure 2a). However, after $Cl^-$-free washes (pH 6.5) without sucrose, a $g = 4$ signal was observed from $S_2$ (Figure 2b). The $S_2$ $g = 4$ signal exhibited a peak to trough width of 34.2 mT and a turning point at $g = 4.2$, showing EPR properties similar to the $S_2$ $g = 4$ signal observed in untreated PS-II in the presence of sucrose (Zimmermann & Rutherford, 1984**,** 1986a). In addition, the intensity of the $S_2$ multiline signal was significantly lowered (see also Figure 4). This was mainly due to a decreased intensity of the hyperfine lines which was 30% of that in control samples, while a broad signal underlying the $S_2$ multiline signal [e.g. Pace et al. (1991)] was apparently unaffected by the $Cl^-$-free washes. Furthermore, in most cases (eight from twelve PS-II preparations used), the $Cl^-$-free washes resulted in some minor changes in the hyperfine structure of the $S_2$ multiline signal (Figure 2b). Although we cannot rule out that a fraction of centers remains unaffected by the $Cl^-$-free washes, the nature of the changes in the multiline signal led us to consider that the majority of centers is modified (i.e. >70% of the centers does not exhibit an $S_2$ multiline signal after $Cl^-$-free washes).

The $S_2$ $g = 4$ and $S_2$ multiline signal in $Cl^-$-free-washed PS-II showed a half-decay time of 4−5 min at room temperature (in the presence of 1 mM PPBQ), similar to that observed from $S_2$ in untreated PS-II under similar conditions [Styring & Rutherford, 1988; see also Brudvig et al. (1983)]. Rapid addition of $Cl^-$ (50 mM) in darkness to the $S_2$ state (Figure 2b), a method that has been used to investigate the $S_2$ state in $Cl^-$-depleted PS-II (Ono et al., 1986), reversed the effects of $Cl^-$-free washes (Figure 2c), resulting in the suppression of the $g = 4$ signal and reconstitution of the multiline signal. The $Q_A^-Fe^{2+}$ EPR signal at $g = 1.90$ (350 mT), detected after generation of $S_2Q_A^-$ by illumination at 200 K (Figure 2a,b), disappeared following thawing of the sample for the addition of $Cl^-$ in darkness (Figure 2c), due to electron tranfer from $Q_A^-$ to PPBQ, resulting in the formation of the semiquinone form of PPBQ. This semiquinone, which is a good oxidant, oxidizes the non-heme iron, giving rise to an EPR signal at $g = 8$ (82 mT) and $g = 6$ (120 mT) (Figure 2c) from $Fe^{3+}$ (Zimmermann & Rutherford, 1986b; Diner & Petrouleas, 1987).

As reported earlier (Zimmermann & Rutherford, 1984, 1986a), when sucrose-containing (0.3−0.5 M) buffers were used, continuous illumination at 200 K of untreated PS-II in the presence of $Cl^-$ (10 mM) resulted in the formation of an $S_2$ $g = 4$ signal in a fraction of centers (not shown). The intensity of this signal was doubled after $Cl^-$-free washes of those samples (not shown). The resulting $S_2$ $g = 4$ EPR signal was similar to that shown in Figure 2b which was recorded in $Cl^-$-free-washed PS-II in the absence of sucrose. The increase of the $S_2$ $g = 4$ signal intensity was reversed by addition of $Cl^-$. Occasionally, $Cl^-$-free-washed PS-II in the absence of sucrose did not exhibit an $S_2$ $g = 4$ signal; otherwise, properties were identical to those described above. When sucrose was added, the $S_2$ $g = 4$ signal was observed to exhibit the $Cl^-$-dependent behavior as described above. These effects of sucrose are not understood but may be related to the binding properties of the extrinsic polypeptides.

Flash illumination of $Cl^-$-free-washed PS-II resulted in a flash-dependent oscillation of the $S_2$ $g = 4$ and multiline signals (Figure 3). The signals from the $S_2$ state oscillated in parallel with maximal intensities on the first and fifth flash. Furthermore, the signal at $g = 8$ originating from the oxidized non-heme iron showed a flash-dependent binary oscillation with maximal intensities on odd-numbered flashes. These results indicate that both the $S_2$ multiline signal and the $S_2$ $g = 4$ signal in $Cl^-$-free-washed PS-II originate from the functional charge accumulation cycle. A flash-dependent

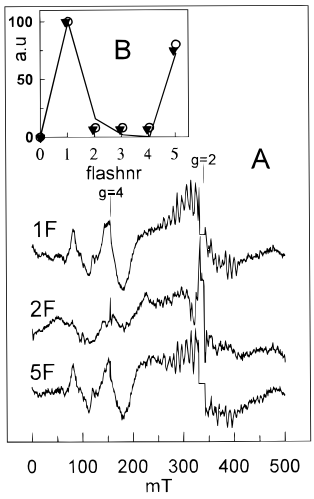


FIGURE 3: (A) Light minus dark EPR spectra of Cl⁻-free washed PS-II membranes in 5 mM MES (pH 6.5) and 0.3 M sucrose, after illumination with single flashes at room temperature in the presence of PPBQ (1 mM), followed by rapid (1 s) freezing in darkness. (B) Intensities of (▼) the signal at $g = 4$ and (○) the multiline signal plotted relative to the number of flashes. The $g = 4$ signal intensity was determined from the peak to trough amplitude, and the multiline signal intensity was determined as the sum of the resolved hyperfine line amplitudes. The continuous line was fitted assuming 100% $S_1$ before illumination, 8% misses, and no double hits. Before addition of PPBQ (1 mM) and flash illumination, the dark-adapted samples were synchronized according to Styring and Rutherford (1988) by illumination with a preflash followed by dark adaptation for 10 min at room temperature. Instrument settings were as in the legend to Figure 2.

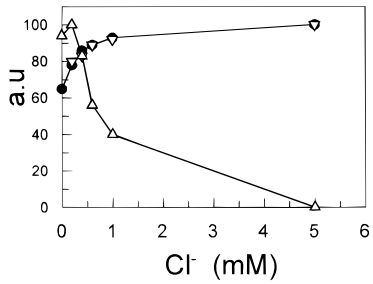


FIGURE 4: Intensities of (△) the $g = 4$ EPR signal and (●) the multiline EPR signal in PS-II membranes after Cl⁻-free washes as described in the legend to Figure 2b, followed by addition on ice of increasing Cl⁻ concentrations to the dark-adapted samples, dark incubation on ice for 30 min, and addition of PPBQ (1 mM). Then the samples were frozen and illuminated at 200 K for 3 min. The $g = 4$ signal intensity was determined as described in the legend to Figure 3. The signal intensity at $g = 2$ was determined as the sum of the resolved hyperfine line amplitudes plus the intensity of the broad signal underlying the hyperfine lines. This was done in samples following rapid thawing (5 s) and freezing in darkness of the illuminated samples, treatment of which resulted in the disappearance of the $Q_A^-Fe^{2+}$ EPR signal at $g = 1.90$ without the signal intensity at $g = 2$ being affected. (▽) Calculated intensities of the signal around $g = 2$. Instrument settings were as in the legend to Figure 2.

oscillation of the $g = 4$ EPR signal was observed previously in untreated PS-II (Zimmermann & Rutherford, 1986a). The results indicate that the Cl⁻ removed at pH 6.3, which affects the $S_2$ EPR properties as described above, is not obligatory for oxygen-evolving activity.

The quantitative relationship between the Cl⁻-dependent intensity of the $S_2$ $g = 4$ signal and that of the $S_2$ multiline signal was studied after reconstitution of increasing Cl⁻ concentrations following the Cl⁻-free washes. The results are shown in Figure 4. We determined the fraction of centers contributing to the EPR signal at around $g = 2$ by addition of the hyperfine line intensities to the intensity of the broad signal underlying the multiline signal. The EPR signal intensity at $g = 2$ in Cl⁻-free-washed PS-II was about 65% of that observed at a Cl⁻ concentration (5 mM) which is sufficient to completely suppress the $g = 4$ signal. The addition of a small amount of Cl⁻ (0.2 mM) resulted in an increased intensity of both the signal at $g = 4$, from 94% to maximal (100%), and the signal at $g = 2$, from 65 to 80%. The origin of this increase of both $S_2$ EPR signals is unknown. It could reflect a small fraction of centers in Cl⁻-free-washed PS-II in which the $S_2$ state was not formed. Alternatively, it is possible that in a fraction of centers the $S_2$ state was reversibly modified by the Cl⁻-free washes and did not exhibit an $S_2$ EPR signal, as is the case after inhibition of oxygen evolution by Cl⁻ depletion of PS-II in the presence of $SO_4^{2-}$ (Ono et al., 1986; Boussac & Rutherford, 1994b).

After addition of higher concentrations of Cl⁻ (>0.2 mM), an inverse relationship was observed between the signal intensities at $g = 4$ and $g = 2$. The relative signal intensities at $g = 2$ calculated from the Cl⁻-dependent decrease of the $g = 4$ signal intensity matched rather well those determined experimentally (Figure 4), indicating that a direct Cl⁻-induced conversion occurred from the $g = 4$ signal to the signal around $g = 2$.

From comparison of the signal intensities at 0.2 mM Cl⁻ [maximal $g = 4$ signal (100%) and 80% $g = 2$ signal] and 5 mM Cl⁻ (no $g = 4$ signal and 100% signal at $g = 2$), it is estimated that following the Cl⁻-free washes about 20% of the centers present in $S_2$ exhibit a $g = 4$ signal, assuming that all centers give rise to an EPR signal. This estimate of the fraction of centers giving rise to the $S_2$ $g = 4$ signal can be considered a lower limit. If the broad signal underlying the multiline EPR signal was excluded from the quantification, a significantly larger fraction (~40%) of centers was estimated to contribute to the $S_2$ $g = 4$ signal.

Cl⁻ depletion treatments in PS-II are often done in the presence of the anions $SO_4^{2-}$ or F⁻ which are thought to enhance Cl⁻ depletion in PS-II [reviewed by Homann (1987)]. However, the addition of $SO_4^{2-}$ (50 mM) or F⁻ (25 mM) to Cl⁻-free-washed PS-II at pH 6.5 had no effect on the EPR properties of $S_2$ (not shown). These anions also did not influence the $S_2$ EPR signals in Cl⁻-free-washed PS-II samples which had been partially reconstituted with Cl⁻ (0.6 mM). Thus, it seems that, under these conditions, the anions $SO_4^{2-}$ and F⁻ did not compete with Cl⁻.

*Effects of Cl⁻ Depletion by Treatment at pH 10.* Treatment of Cl⁻-free-washed PS-II at high pH (pH 10) resulted in extensive inhibition of oxygen evolution. The residual oxygen-evolving activity during the measurement was lost relatively rapidly with a half-inhibition time of 24 s and showed an initial rate of about 15% relative to that after reconstitution with 10 mM Cl⁻. The Cl⁻-reconstituted oxygen-evolving activity in pH 10-treated PS-II was about 90% relative to that of Cl⁻ reconstituted, Cl⁻-free-washed PS-II, indicating 10% irreversible inhibition following Cl⁻ depletion by pH 10 treatment, probably due to a fraction of damaged centers. The initial rate of Cl⁻-reconstituted oxygen evolution remained approximately constant for about 2 min, in a fashion similar to that in untreated and Cl⁻-free-washed PS-II. Furthermore, the Cl⁻ affinity for reconstitution of oxygen-evolving activity in pH 10/Cl⁻-depleted PS-II was

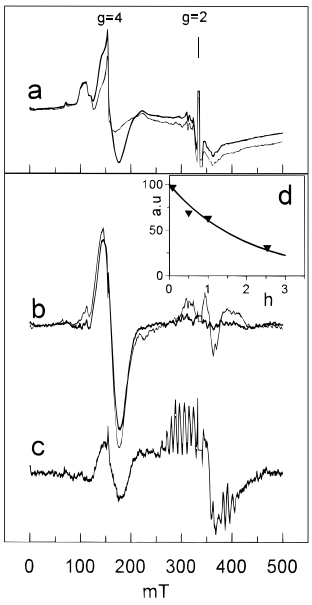


FIGURE 5: (a) EPR dark spectra of PS-II (thin) prior to or (thick) following pH 10/$Cl^-$ depletion treatment recorded after dark adaptation for 5 min on ice. (b) EPR difference spectra of pH 10/$Cl^-$-depleted PS-II (thick) after 5 min of dark adaptation or (thin) after illumination at 200 K for 4 min after subtraction of the dark base line spectrum [a (thin)] of the sample prior to the pH 10 treatment. (c) Light minus dark EPR spectrum following pH 10/$Cl^-$ depletion and reconstitution with 0.2 mM $Cl^-$ after illumination at 200 K for 3 min. (d) Intensities of the signal around $g = 4$ in pH 10/$Cl^-$-depleted PS-II (▼) after increasing periods of dark incubation on ice. The PS-II membranes were resuspended in 10 mM MES (pH 6.3) and 0.5 M sucrose. The intensities of the spectra from b and c were multiplied by 2.5 in comparison to the intensity of the spectra from a. Instrument settings were as in the legend to Figure 2.

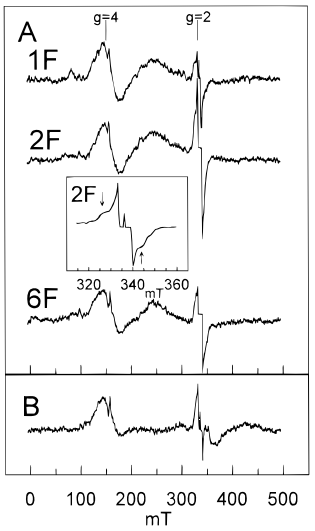


FIGURE 6: Light minus dark EPR spectra of pH 10/$Cl^-$-depleted PS-II after illumination with (A) single flashes or (B) continuous illumination at 0 °C followed by rapid freezing. The inset shows the light-induced signal around $g = 2$ generated after illumination with two flashes, and the arrows indicate the peak to trough width of the resolved component. Before the illumination, the samples were dark adapted at room temperature for 35 min, followed by addition of ferricyanide (50−100 $\mu$M) and PPBQ (50−100 $\mu$M). Instrument settings were as in the legend to Figure 2.

relatively high, with 50% reconstitution of oxygen evolution at a $Cl^-$ concentration of about 100 $\mu$M (not shown). This value for the $Cl^-$ binding constant is in agreement with those determined earlier in similarly treated PS-II and is taken as an indication that the 17 and 23 kDa extrinsic polypeptides are associated to most of the pH 10/$Cl^-$-depleted PS-II centers (Homann, 1985, 1988a, 1993). This was confirmed by SDS gel electrophoresis and subsequent Western blotting (not shown).

Surprisingly, after pH 10/$Cl^-$ depletion, an EPR signal around $g = 4$ was observed in the samples that were dark adapted for 5 min [Figure 5a (thick)] [compare to the $Cl^-$-free-washed (pH 6.3) sample [Figure 5a (thin)]]. This signal, which was presumably photogenerated during the pH 10/$Cl^-$ depletion treatment done in dim light, was lost upon longer dark adaptation (Figure 5d) and could be regenerated by continuous illumination at 200 K [Figure 5b (thin)] or by illumination with a single flash at room temperature (Figure 6), indicating that the $g = 4$ signal observed after pH 10 treatment corresponds to the $S_2$ state. However, no $S_2$ multiline was observed before or after the illumination treatments (Figures 5a,b and 6). A suppression of the $S_2$ multiline signal also has been observed after $Cl^-$ depletion in PS-II in the presence of various anions [see Ono et al. (1987)]. The $S_2$ $g = 4$ signal observed in pH 10/$Cl^-$-depleted PS-II exhibited EPR properties similar to those of the $S_2$ $g = 4$ signal in $Cl^-$-free-washed PS-II (Figure 2b). However, the lifetime of the $S_2$ state in pH 10/$Cl^-$-depleted PS-II giving rise to the $g = 4$ signal is clearly longer than that in $Cl^-$-free-washed PS-II since 5 min of dark adaptation on ice had little effect on the $S_2$ $g = 4$ signal observed after pH 10/$Cl^-$ depletion [Figure 5a (thick), Figure 5b)], while in $Cl^-$-free-washed PS-II, the $S_2$ $g = 4$ signal was absent [Figure 5a (thin)]. The half-decay time of the $S_2$ $g = 4$ signal in pH 10/$Cl^-$-depleted PS-II was about 1.5 h at 0 °C (Figure 5d) and approximately 10 min at room temperature (not shown). No $Q_A^-Fe^{2+}$ EPR signal was present in the EPR spectrum of the short dark-adapted pH 10/$Cl^-$-depleted PS-II samples [Figure 5a,b (thick)]. Thus, it is very unlikely that the decay of the long-lived $g = 4$ signal in pH 10/$Cl^-$-depleted PS-II is due to a recombination reaction with $Q_A^-$.

In the presence of the external electron acceptor PPBQ (1 mM), the decay of the $S_2$ $g = 4$ signal was accelerated. This is probably due to reduction of the Mn cluster by $PPBQH_2$, leading to some $Mn^{2+}$ release, as indicated by the appearance of a six-line signal around $g = 2$ in the EPR spectrum, originating from hexaquomanganese(II) (not shown).

The intensity of the $S_2$ $g = 4$ signal in pH 10/$Cl^-$-depleted PS-II was markedly enhanced, showing an intensity of 2−3 times that observed in $Cl^-$-free-washed PS-II (Figure 2b). From the estimate of the fraction of centers (20−40%) in $Cl^-$-free-washed PS-II giving rise to the $S_2$ $g = 4$ signal (Figures 2b and 4), it is estimated that the $S_2$ $g = 4$ signal observed in pH 10/$Cl^-$-depleted PS-II corresponds to 40−80% of the centers.

The effects of pH 10/$Cl^-$ depletion on the EPR properties of $S_2$ were reversed after addition of $Cl^-$ (for the experimental conditions, see Materials and Methods). No $S_2$ EPR signals were observed after short dark adaptation on ice. After illumination at 200 K of the dark-adapted $Cl^-$-reconstituted PS-II, a normal light-induced $S_2$ EPR spectrum, which is dominated by the presence of a characteristic $S_2$ multiline signal, was observed (Figure 5c). The extent to which the $S_2$ multiline signal intensity in pH 10/$Cl^-$-depleted PS-II was reconstituted with $Cl^-$ was comparable to the $Cl^-$-reconstituted level of oxygen-evolving activity under these conditions; i.e.

addition of 0.2 mM $Cl^-$ after pH 10 treatment (Figure 5c) resulted in reconstitution of the $S_2$ multiline signal and the oxygen-evolving activity to about 80% of those determined after addition of a high concentration of $Cl^-$ (20 mM).

Dark adaptation of the pH 10/$Cl^-$-depleted PS-II samples at room temperature for 35 min resulted in the decay of the $S_2$ $g = 4$ signal in most of the centers. After this dark adaptation treatment, most of the centers were in $S_1$ since, after addition of $Cl^-$ (50 mM) to these samples, a regular $S_2$ multiline signal was generated with continuous illumination at 200 K or illumination with a single flash at room temperature (not shown, but see Figure 5c).

Illumination of dark-adapted pH 10/$Cl^-$-depleted PS-II with a single flash resulted in the formation of the $S_2$ $g = 4$ signal (Figure 6A). Following illumination with two flashes, the $S_2$ $g = 4$ signal intensity was similar to that observed after one flash. In addition, a narrow signal around $g = 2$ was generated, corresponding to the formal $S_3$ state (Figure 6A). Part of this signal shows a peak to trough width of about 16.4 mT [Figure 6 (inset)]. The width of this part of the signal was comparable to the $S_3$ signal observed in $Ca^{2+}$-depleted PS-II (Boussac & Rutherford, 1989). In addition, a narrower component (<10 mT) of the $S_3$ signal was present but was unresolved due to the presence of the $TyrD^{\bullet}$ radical signal [Figure 6 (inset)]. A narrow (<10 mT) $S_3$ signal has been observed in $Cl^-$-depleted PS-II following treatment with $SO_4^{2-}$ (Boussac et al., 1992) or $F^-$ (Baumgarten et al., 1990). No $S_3$ signal could be generated following $Cl^-$ reconstitution in pH 10/$Cl^-$-depleted PS-II (not shown) which confirmed that the ability to generate the $S_3$ EPR signal was a $Cl^-$ depletion effect (and not the result of inadvertant $Ca^{2+}$ depletion occurring in addition to $Cl^-$ depletion).

Following illumination with multiple flashes (Figure 6A) or with continuous illumination at 0 °C (Figure 6B), the intensity of the $g = 4$ signal was only slightly smaller than that observed after one flash. The EPR signal attributed to $S_3$ was decreased after the illumination treatments but was still detected under these conditions. The significance of this decrease is unknown; however, a similar decrease in the intensity of the $S_3$ signal was recently observed in $Ca^{2+}$-depleted PS-II upon multiple flash illumination (Boussac & Rutherford, 1995).

The results indicate that at least a fraction of pH 10/$Cl^-$-depleted PS-II centers gives rise to the $S_3$ signal after two flashes. The $S_3$ to $S_0$ transition in these centers seemed to be largely inhibited as is the case after $Cl^-$ depletion of PS-II by treatment with $SO_4^{2-}$ (Boussac et al., 1992) or $F^-$ (Baumgarten et al., 1990). Nevertheless, the observation that the $S_2$ $g = 4$ signal is rather insenstive to a second flash or further illumination treatments raises the question whether the $S_2$ $g = 4$ and $S_3$ EPR signals originate from common or different centers. Relevant to this question is the observation (not shown) that the $g = 4$ signal in pH 10/$Cl^-$-depleted PS-II was much smaller when illumination treatments were done in the presence of high concentrations (1 mM) of PPBQ. In contrast, the $S_3$ signal intensity was significantly less affected by PPBQ. This indicates that the centers exhibiting the $S_2$ $g = 4$ signal were different from those in which an $S_3$ signal was generated. The effect of a high concentration of PPBQ is tentatively attributed to the reduction of the Mn cluster in the centers exhibiting the $g = 4$ signal, presumably due to the presence of $PPBQH_2$. In fact, the experiments from Figure 6 were done using low concentrations of PPBQ (50−100 $\mu$M) in the presence of ferricyanide (50−100 $\mu$M) to avoid such an effect.

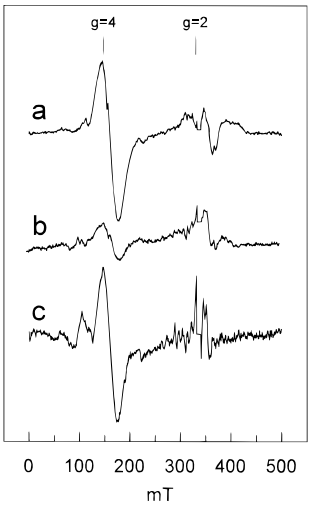


FIGURE 7: EPR difference spectra of PS-II after (a) pH 10/$Cl^-$ depletion (see Figure 5b) followed by (b) addition of $SO_4^{2-}$ (20 mM) or (c) addition of $F^-$ (25 mM) as described in Materials and Methods and resuspension in 10 mM MES (pH 6.3) and 0.5 M sucrose. The spectra were recorded after illumination at 200 K for 4 min followed by subtraction of the dark base line spectrum recorded prior to pH 10/$Cl^-$ depletion [see Figure 5a (thin)]. Instrument settings were as in the legend to Figure 2.

*Effects of $SO_4^{2-}$ and $F^-$ in pH 10/$Cl^-$-Depleted PS-II.* $Cl^-$ depletion in PS-II is often done in the presence of the counteranions $SO_4^{2-}$ [see Ono et al. (1986)] or $F^-$ [see Baumgarten et al. (1990)] which are thought to enhance $Cl^-$ depletion from PS-II (Homann, 1987). The $S_2$ multiline signal intensity has been reported to diminish after $Cl^-$ depletion of PS-II in the presence of these anions [see Ono et al. (1986) and Baumgarten et al. (1990)]. In addition, the anion $F^-$ seems to specifically enhance the $S_2$ $g = 4$ signal [see Ono et al. (1987) and DeRose et al. (1995)]. Figure 7 shows the effects of addition (at pH 7.3) of $SO_4^{2-}$ (20 mM) or $F^-$ (25 mM) on the EPR properties of $S_2$ in pH 10/$Cl^-$-depleted PS-II, detected at pH 6.3. In comparison to the control sample (Figure 7a, see also Figure 5a,b), the anion $SO_4^{2-}$ largely inhibited detection of the $S_2$ $g = 4$ signal (Figure 7b). The anion $F^-$, however, had little effect under these conditions (Figure 7c).

Several studies on $Cl^-$-depleted PS-II reported in the literature were done at pH 7.5 [see Ono et al. (1986) and Beck and Brudvig (1988)]. Figure 8 shows the effect of $F^-$ (25 mM) in pH 10/$Cl^-$-depleted PS-II detected at pH 7.3. The $S_2$ $g = 4$ signal of the $F^-$-treated sample detected at pH 7.3 (Figure 8b) was similar to that detected at pH 6.3 (Figures 7a and 5b). However, in the absence of $F^-$, the $S_2$ $g = 4$ signal intensity at pH 7.3 (Figure 8a) was only 50% of that at pH 6.3. A similar decrease of the $S_2$ $g = 4$ signal intensity was observed (not shown) after the pH in the samples was increased from pH 6.3 (see Figure 7a) by addition of 100 mM HEPES (pH 7.3). Thus, the $S_2$ $g = 4$ signal intensity in pH 10/$Cl^-$-depleted PS-II is pH-dependent and increases at lower pH. The anion $F^-$ affected the $S_2$ EPR properties in $Cl^-$-depleted PS-II in a fashion similar to lowering the pH. This effect of $F^-$ (25 mM) also was observed (not shown) when $SO_4^{2-}$ (20 mM) was present. Thus, the effect of $SO_4^{2-}$ and increased pH on the EPR properties of $S_2$, resulting in the suppression of the $g = 4$ signal, was

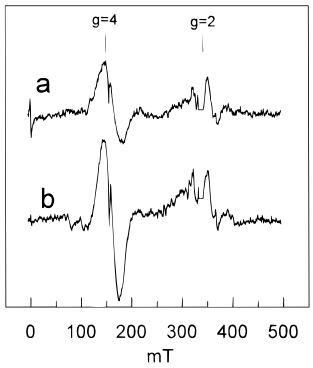


FIGURE 8: EPR difference spectra of pH 10/$Cl^-$-depleted PS-II as in Figure 7 except that after the pH 10 treatment the pH was lowered to pH 7.3 instead of pH 6.3 and the resuspension solution was buffered with 10 mM HEPES (pH 7.3). (a) No additions or (b) following addition of $F^-$ (25 mM) in the dark.

overridden by $F^-$. Furthermore, at an increased pH, in the absence or presence of $F^-$, the lifetime of the $S_2$ $g = 4$ signal in pH 10/$Cl^-$-depleted PS-II was similar to that determined at pH 6.3. The results indicate that the anions $SO_4^{2-}$ and $F^-$ have distinct effects on the $S_2$ EPR properties of pH 10/$Cl^-$-depleted PS-II, resulting in $S_2$ EPR properties similar to those observed after $Cl^-$ depletion in PS-II using these anions as counterions [see Boussac and Rutherford (1994b) and Ono et al. (1987)].

The similarities between the $S_2$ $g = 4$ signals observed in the absence and presence of $F^-$ in pH 10/$Cl^-$-depleted PS-II samples led us to test if the $S_2$ $g = 4$ signal in the presence of $F^-$ showed the same response to flash illumination as that in the absence of $F^-$ (Figure 6). The results are shown in Figure 9. After illumination of the $F^-$-treated (25 mM), pH 10/$Cl^-$-depleted PS-II with a single flash, an $S_2$ $g = 4$ signal was generated which was similar to that in the absence of $F^-$. After the second flash, the $S_2$ $g = 4$ signal intensity in the $F^-$-treated sample was significantly decreased, indicating that an $S_2$ to $S_3$ transition occurred in most of the centers that exhibited the $S_2$ $g = 4$ signal. In addition, a narrow signal around $g = 2$ corresponding to the formal $S_3$ state was generated (Figure 9), similar to that observed in pH 10/$Cl^-$-depleted PS-II in the absence of $F^-$ (Figure 6). Illumination with further flashes indicated that the $g = 4$ signal in the $F^-$-treated sample shows a damped, flash-dependent oscillation with maximal intensities on the first and the fifth flash (Figure 9). In addition, although noisy and poorly resolved due to the presence of the dark stable TyrD$^\bullet$ radical signal, the $S_3$ signal also showed a significant degree of a damped, flash-dependent oscillation with maximal intensities on the second and sixth flash (Figure 9).

The presence of oscillating EPR signals (albeit damped) in $F^-$-treated pH 10/$Cl^-$-depleted PS-II indicates enzyme turnover. This was confirmed by measurements of oxygen evolution. A study of the oxygen-evolving activity versus light intensity (Figure 1) indicated that about 45% of the centers in $F^-$-treated, pH 10/$Cl^-$-depleted PS-II (Figure 1, open triangles) were evolving oxygen but were slowed in the enzyme turnover by about a factor of 2 (in comparison to $Cl^-$-reconstituted, $Cl^-$-free-washed PS-II, Figure 1, open circles). Thus, the results indicate that $F^-$ functionally replaced $Cl^-$ to a large extent. The halides $I^-$ (Rashid & Homann, 1992) and $Br^-$ (Hind et al., 1969) also are known to functionally replace $Cl^-$.

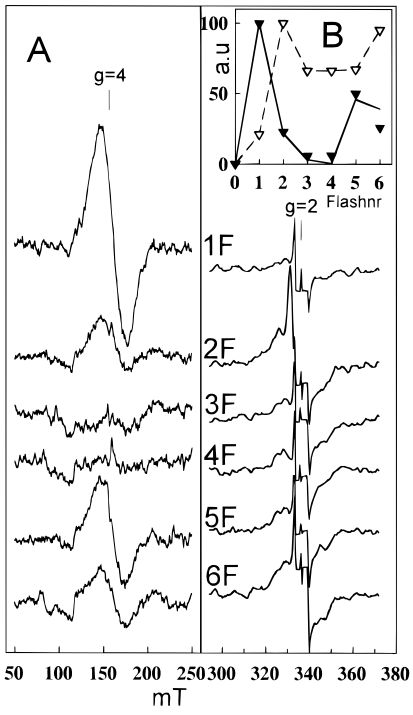


FIGURE 9: (A) Light minus dark spectra of pH 10/$Cl^-$-depleted PS-II after reconstitution with $F^-$ (25 mM) and illumination with single flashes as in Figure 6 in the presence of ferricyanide (50−100 $\mu$M) and PPBQ (50−100 $\mu$M). (B) Intensities of (▼) the $g = 4$ signal determined as in Figure 2 and (--▽--) the signal around $g = 2$ determined by double integration of the light-induced spectrum, plotted relative to the number of flashes. The continuous line was fitted assuming 10% $S_0$ and 90% $S_1$ before illumination and 6% misses on the S-state transitions except for the $S_3$ to $S_0$ transition which was assumed to be accompanied by 45% misses (see also Discussion). The PS-II membranes were resuspended in 10 mM MES (pH 6.3), 0.5 M sucrose, and 25 mM $F^-$. Instrument settings were as in the legend to Figure 2.

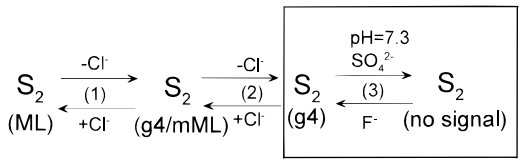


FIGURE 10: Schematic representation of the effects of $Cl^-$ depletion treatments and the effects of anions on the EPR properties of $S_2$ in PS-II. ML = regular multiline signal. mML = multiline signal with spectral modifications. g4 = signal around $g = 4$. The steps are as follows. (1) $Cl^-$-free washes of PS-II at pH 6.3−6.5/$Cl^-$ reconstitution (Figures 1−4). This treatment has a small effect on the enzyme kinetics. (2) $Cl^-$ depletion of PS-II by treatment at pH 10, resulting in inhibition of oxygen evolution and a mixture of two types of centers as indicated in the box (Figures 5a,b, 6, and 7a) which was either reversed by $Cl^-$ reconstitution (Figure 5c) or (3) further influenced by the pH (Figure 8a), $SO_4^{2-}$ (Figure 7b), and $F^-$ (Figures 7c and 8b). It is of note that, in the presence of $F^-$, the $S_2$ $g = 4$ signal in pH 10/$Cl^-$-depleted PS-II originates from centers which are functionally different from those in the absence of $F^-$ (see text).

## DISCUSSION

The results indicate that PS-II exhibits two distinct $Cl^-$-dependent properties. These and further observations described in the results are depicted in Figure 10. The distinct $Cl^-$-dependent properties of PS-II are most straightforwardly explained by considering the presence of two $Cl^-$ sites in PS-II, one of which is essential for oxygen-evolving activity.

Some effects of salts on the functional properties of the oxygen-evolving complex have been suggested to arise from conformational changes of PS-II by alterations of hydrophobic and/or electrostatic interactions on the protein surface of PS-II [Wydrzynski et al., 1990; Pauly et al., 1992; see also Kebekus et al. (1995)]. In general, such salt-induced effects on proteins occur at relatively high salt concentrations, ranging from tens of millimolar to molar concentrations [see von Hippel and Schleich (1969) and Aviram (1973)]. The $Cl^-$-dependent properties of PS-II observed at pH 6.3 could originate from such salt effects. However, reconstitution of the $S_2$ multiline signal at the expense of the $S_2$ $g = 4$ signal occurred at a relatively low $Cl^-$ concentration, with an apparent $Cl^-$ affinity of ≈0.8 mM (Figure 4). Moreover, in contrast to $Cl^-$, the anions $SO_4^{2-}$ (50 mM) and $F^-$ (25 mM) added under similar conditions did not affect the $S_2$ EPR properties in $Cl^-$-free-washed PS-II. Thus, the $Cl^-$-dependent properties of PS-II at pH 6.3 (Figures 1−4) point to the presence of a $Cl^-$-binding site in PS-II which is not essential for oxygen evolution.

The oxygen-evolving activity measured as a function of light (Figure 1) indicated that depletion of the $Cl^-$ not essential for oxygen evolution at pH 6.3 inhibited the formation of the enzyme−substrate complex (Hofstee, 1952). Since in this case light is considered to be the substrate, the results indicate that the removal of $Cl^-$ at pH 6.3 lowered the quantum yield of water oxidation, reflecting the inhibition of any reversible process that is involved in charge accumulation. A slow down of electron transfer, allowing charge recombination or enhanced deactivation of the higher S states, might be considered as possibilities.

The depletion of $Cl^-$ from PS-II by high-pH (pH 10) treatment resulted in only a residual oxygen-evolving activity (see Results). It is considered that this reflects $Cl^-$ depletion from the $Cl^-$ site in PS-II that has been studied in previous work on $Cl^-$-depleted PS-II (Debus, 1992). This site probably corresponds to the slowly exchanging 1 $Cl^-$/PS-II identified by Lindberg et al. (1990, 1993) that was detected from measurements of $Cl^-$ release from $^{36}Cl^-$-labeled PS-II at pH 6.3. The measurements by Lindberg et al. (1993) were done after removal of the $Cl^-$ from PS-II at pH 6.3. This pretreatment is essentially similar to the $Cl^-$-free washes used in this work. Thus, the other $Cl^-$ site, which is not obligatory for oxygen evolution (see above), would not have been detected by Lindberg et al. (1993). After complete release of the $^{36}Cl^-$ from PS-II, Lindberg et al. [(1993), Figure 5] observed significant oxygen-evolving activity (30%). The apparent lack of correlation between the slowly exchanging $Cl^-$ and oxygen evolution implied that the $Cl^-$ released was not required for oxygen evolution (Lindberg et al., 1993). However, the observation by Lindberg et al. (1993) of a residual oxygen-evolving activity after long incubation is consistent with their earlier study (Lindberg et al., 1990) in PS-II isolated from spinach grown using $^{36}Cl^-$-containing nutrients, which indicated the presence of a fraction of centers (30%) containing $Cl^-$ which was not exchangable by incubation at pH 6.3. Since Lindberg et al. (1993) used similar incubation conditions for $^{36}Cl^-$ binding to normal PS-II membranes, it is very likely that the remaining oxygen-evolving activity after complete release of $^{36}Cl^-$ from PS-II [Lindberg et al. (1993), Figure 5] corresponds to the fraction of unlabeled centers containing the nonexchangable $Cl^-$. Thus, the most straightforward explanation of the results obtained by Lindberg et al. (1990, 1993) is that the slowly exchanging 1 $Cl^-$/PS-II is essential for oxygen-evolving activity.

The $S_2$ $g = 4$ signal in pH 10/$Cl^-$-depleted PS-II (Figure 5) was rather stable ($t_{1/2} \approx 1.5$ h at 0 °C), unlike that observed in $Cl^-$-free-washed PS-II. This may reflect a lowered oxidation potential of $S_2$ after the pH 10/$Cl^-$ depletion treatment. A greater stability of $S_2$ in pH 10/$Cl^-$-depleted PS-II was also manifested as an upshifted emission temperature of the thermoluminescence from recombination of $S_2Q_A^-$ and $S_2Q_B^-$ (Rashid & Homann, 1992; Homann, 1993).

The state giving rise to the $S_2$ $g = 4$ EPR signal in pH 10/$Cl^-$-depleted PS-II (≥40% of the centers) was rather insensitive to a second flash or further illumination treatments (Figure 6). Nevertheless, a fraction of centers exhibited a narrow signal around $g = 2$ (Figure 6) corresponding to the formal $S_3$ state (Figure 6). The results indicated that this fraction of centers is distinct from that exhibiting the $S_2$ $g = 4$ signal. This implies that, in the centers exhibiting the $S_2$ $g = 4$ signal (Figures 5 and 6), the $S_2$ to $S_3$ transition was blocked. Thus, the $S_2$ state in the fraction of centers that was able to advance to $S_3$ probably corresponds to a state that was not detected by EPR, as is the case after $Cl^-$ depletion of PS-II in the presence of $SO_4^{2-}$ (Ono et al., 1986; Boussac et al., 1992; Boussac & Rutherford, 1994b).

The results showed that the addition of the anions $SO_4^{2-}$ and $F^-$ to pH 10/$Cl^-$-depleted PS-II affected the $S_2$ $g = 4$ signal intensity (Figures 7 and 8) and resulted in $S_2$ EPR properties which were similar to those observed after $Cl^-$ depletion in PS-II using these anions as counterions [see Boussac and Rutherford (1994b) and Ono et al. (1987)]. These effects and those of the pH (Figure 8) probably reflect alterations in the relative contributions of the states giving rise to an $S_2$ $g = 4$ signal and the "EPR-undetected" $S_2$ state (Figure 10). Boussac and Rutherford (1994b) have proposed that generation of the modified, EPR-undetected $S_2$ state in $Cl^-$-depleted PS-II is accompanied by oxidation of an amino acid residue instead of Mn oxidation, due to a $Cl^-$-dependent redox equilibrium between the Mn cluster and a nearby amino acid residue. In the context of this model, it can be speculated that this redox equilibrium is affected by the pH and the presence of other anions.

The addition of $F^-$ to pH 10/$Cl^-$-depleted PS-II resulted in reconstitution of the oxygen-evolving activity in almost 50% of the centers. However, the enzyme turnover in the $F^-$-reconstituted centers is significantly slowed relative to the control sample (Figure 1), resulting in a "residual" oxygen-evolving activity (under nearly saturating light) of ≈25% relative to that after reconstitution with $Cl^-$. Such residual oxygen-evolving activities in $F^-$-reconstituted PS-II have been reported earlier (Casey & Sauer, 1984; Baumgarten et al., 1990; Homann, 1993; DeRose et al., 1995). It is quite possible that, in these studies, reconstitution effects of $F^-$ similar to those observed in this work occurred but were not detected since the oxygen-evolving activity was not measured as a function of the light intensity.

The presence of a significant fraction of functional centers in $F^-$-reconstituted PS-II also was indicated by the flash-dependent (albeit damped) oscillation of the $S_2$ $g = 4$ EPR signal (Figure 9) and the narrow EPR signal around $g = 2$, corresponding to the formal $S_3$ state (Figure 9). The results from fitting of the oscillating EPR signals from $F^-$-reconstituted PS-II were consistent with those from the

light-dependent oxygen evolution versus light-intensity study (Figure 1) which indicated a fraction (≈45%) of functional centers in which the enzyme turnover was slowed and a fraction (≈55%) of inactive centers. (1) The oscillation pattern of the $g = 4$ signal was best fit by assuming a relatively high miss factor of 40−45% either on the $S_3$ to $S_0$ or on the $S_0$ to $S_1$ transition (Figure 9) which may suggest that the enzyme turnover is rate-limiting on either of these transitions. (2) Although it is not very clear to what extent the functional and inactive centers contribute to the $S_3$ EPR signal, reasonable fits to the flash-dependent $S_3$ signal intensities were obtained by assuming that a significant fraction (50−60%) of centers was turning over while the other fraction was blocked upon formation of $S_3$ (not shown). Further characterization of the enzyme turnover of $F^-$-reconstituted PS-II requires measurements of flash-dependent oxygen evolution.

$S_3$ EPR signals are typically observed following inhibition of oxygen evolution by $Ca^{2+}$ depletion treatments [e.g. Boussac et al. (1989)] or 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 (McLachlan et al., 1993) which are thought to displace the functional $Cl^-$ in PS-II. Recently, however, an $S_3$ signal also has been reported in functional oxygen-evolving PS-II in which $Ca^{2+}$ and $Cl^-$ were replaced by $Sr^{2+}$ and $Br^-$, resulting in a slowing of the enzyme turnover by a factor of 4 (A. Boussac, presented at the ESF workshop Oxygen Evolution, Gif-sur-Yvette, France, November 1994). The flash-dependent oscillation of the $S_3$ EPR signal intensity observed in the present study in $F^-$-reconstituted PS-II seems to indicate that the $S_3$ EPR signal represents a transient intermediate involved in forward electron transfer or a side path component, which is oxidized in a redox equilibrium with the Mn cluster [e.g. Boussac and Rutherford (1994b)]. Since $Cl^-$ seems to be required for proton release (Boussac et al., 1992; Lübbers et al., 1993) in PS-II, we speculate that the appearance of an $S_3$ EPR signal is related to an inhibited or a slowed deprotonation event.

Several reports have indicated that, after removal of the 17 and 23 kDa extrinsic polypeptides by salt washing, no or little $g = 4$ signal can be generated from $S_2$ (de Paula et al., 1986; Boussac & Rutherford, 1988b; van Vliet et al., 1994). Thus, the question arises whether the fraction (<60%) of centers exhibiting no signal from $S_2$ after pH 10/$Cl^-$ depletion treatment lost the extrinsic polypeptides. Protein analysis by SDS gel electrophoresis and subsequent Western blotting following $Cl^-$-free washes at pH 6.3, pH 10/$Cl^-$ depletion treatment, and further incubation with $F^-$ (25 mM) (see above) indicated the presence of the extrinsic polypeptides in nearly all the centers (not shown). Incubation of pH 10-treated PS-II with $SO_4^{2-}$ (20 mM), however, resulted in a partial loss of the extrinsic polypeptides (not shown), i.e. 40% loss of the 23 kDa and 70% loss of the 17 kDa polypeptide [see also Homann (1988c), Beauregard and Popovic (1988), Homann (1992), and Lindberg et al. (1993)]. Thus, no correlation was observed between the $S_2$ EPR properties in pH 10-treated PS-II and the presence/absence of the extrinsic polypeptides. However, it cannot be excluded that the two types of centers in pH 10-treated PS-II exhibiting either the $S_2$ $g = 4$ signal or no signal from $S_2$ reflect differences in the nature of binding of the extrinsic polypeptides to PS-II.

Figure 10 summarizes the conclusions from this work. Two $Cl^-$ sites are considered to be present in PS-II. One of the sites is not essential for oxygen evolution and was previously ignored. This site is depleted of $Cl^-$ by $Cl^-$-free washes at pH 6.3 (Figure 10, step 1), resulting in a modified structure of the Mn cluster and a lowered quantum yield of water oxidation. A second $Cl^-$ site is essential for oxygen evolution and is equivalent to that studied in previous work on $Cl^-$-depleted PS-II. Depletion of $Cl^-$ from this site by treatment at pH 10 (Figure 10, step 2) results in two types of centers exhibiting either a $g = 4$ signal or no signal from the $S_2$ state. The $S_2$ $g = 4$ signal shows an increased stability which may indicate that the oxidation potential of the $S_2$ state is lowered. The fraction of centers exhibiting the $S_2$ $g = 4$ signal appears to be blocked on the $S_2$ to $S_3$ transition. A second and perhaps smaller fraction of centers exhibits no $S_2$ EPR signal and is inhibited on the $S_3$ to $S_0$ transition. After formation of the formal $S_3$ state in these centers, a narrow signal around $g = 2$ is observed. The distribution of the two types of centers in pH 10/$Cl^-$depleted PS-II is further influenced by the pH, and the anions $SO_4^{2-}$ and $F^-$ (Figure 10, step 3), resulting in EPR properties of the $S_2$ state similar to those previously observed after $Cl^-$ depletion of PS-II in the presence of these anions. These effects may be explained in the context of a model proposed earlier (Boussac & Rutherford, 1994b) by a redox equilibrium between the Mn cluster and a nearby amino acid residue which is influenced by anions. The anion $F^-$ is able to occupy the $Cl^-$ site essential for oxygen evolution, resulting in reconstitution of oxygen-evolving activity in a significant fraction of centers. The $S_2$ $g = 4$ signal in $F^-$-reconstituted PS-II shows a damped flash-dependent oscillation and thus originates from centers which differ from those in the absence of $F^-$.

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