Nature of the Inhibition of the Oxygen-Evolving Enzyme of Photosystem II Induced by NaCl Washing and Reversed by the Addition of Ca^{2+} or Sr^{2+} †

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ABSTRACT: The inhibition of the oxygen-evolving enzyme of photosystem II, induced by NaCl washing, has been studied by monitoring the yield of the EPR multiline signal arising from the S_2 charge storage state. After continuous illumination at 200 K, the S_2-multiline signal was present with almost the same amplitude in inhibited and Ca^{2+}-reconstituted membranes. Flash illumination given at room temperature to Ca^{2+}-reconstituted membranes resulted in the usual period four oscillation pattern in the amplitude of the S_2-multiline signal. In the absence of Ca^{2+}, although the multiline signal was formed on the first flash and decreased on the second flash, no further increase in the signal amplitude occurred on the fifth flash. In addition, dark adaptation for 15 s of inhibited membranes that had been given three flashes resulted in the formation of the S_2-multiline signal due to deactivation of the S_3 state. These results indicate that in NaCl-washed material inhibition of S-state turnover occurs at the S_3 to S_0 transition. These results disagree with several earlier reports. Attempts have therefore been made to reconcile these reports with the present work both experimentally and by reevaluation of the earlier data. Other apparent inconsistencies in the literature are discussed in terms of differential depletion of Ca^{2+} from sites with different binding affinities. We speculate that these complex phenomena may be simply explained within a model in which a single Ca^{2+} specific binding site is associated with oxygen evolution and that the affinity of this site is modulated by the S states. It is also shown that reactivation by Sr^{2+} instead of Ca^{2+} induced a modified multiline signal similar to that observed in NH_3-treated photosystem II membranes.

The oxidation of a pair of water molecules and the resultant oxygen evolution by photosystem II (PS-II)[1] of plants involve a cluster of four Mn atoms linked to the reaction center complex and three membranous extrinsic polypeptides with molecular masses of 18, 24, and 33 kDa [see Murata and Miyao (1985) for a review]. During this process the donor side of PS-II goes through five different redox states that are denoted S_n, n varying from 0 to 4 according to the model of Kok et al. (1970). The four charges are probably stored on the Mn cluster [see Brudvig (1987) for a review]. Some of the functions of the extrinsic proteins have been determined by studying the effects of their selective removal and rebinding in purified PS-II membranes. The 33-kDa protein stabilizes the Mn cluster in its site (Ono & Inoue, 1983a; Kuwabara et al., 1985), and its absence results in oxygen being released at a slower rate (Miyao et al., 1987). The 24- and 18-kDa proteins act as concentrators of the two cofactors Ca^{2+} and Cl^- (Murata & Miyao, 1985) and shield the Mn cluster from exogenous reductants that can induce Mn release from the membrane (Ghanotakis et al., 1984b).

The requirement of Cl^- for oxygen evolution is well demonstrated [reviewed by Critchley (1985)]; however, its molecular mechanism is not known. Nevertheless, it has been shown that in the absence of Cl^- the S_2 state is abnormal and, at least in the presence of the sulfate counterion, the $S_2 \rightarrow S_3$ transition is inhibited (Theg et al., 1984; Itoh et al., 1984; Ono et al., 1986).

Evidence for a role of Ca^{2+} in PS-II, particularly associated with oxygen evolution, has been accumulated over several years [reviewed by Brand and Becker (1984)]. It is now clear that there are Ca^{2+} requirements for a number of different processes on the donor side of PS-II.

Ca^{2+} plays a role in the photoactivation process (Ono & Inoue, 1983b; Pistorius & Schmid, 1984) by which Mn ions are assembled in the oxygen-evolving enzyme (Tamura & Cheniae, 1987) in an appropriate redox state. The role of Ca^{2+} in the photoactivation process could be related to the observation that Ca^{2+} binding is required for evolution of oxygen in the dark from H_2O_2 via a cyclic reaction involving the S_{-1} and S_1 states (Frash & Mei, 1987).

In cyanobacteria, Ca^{2+} seems to be required for an electron-transfer step closer to the reaction center than the S states (Brand & Becker, 1984; Satoh & Katoh, 1985). A role for Ca^{2+} in S-state turnover probably also exists in cyanobacteria (England & Evans, 1983). In higher plants, Ca^{2+} has been shown to restore oxygen evolution after the release of the 18- and 24-kDa proteins (Ghanotakis et al., 1984a; Miyao & Murata, 1984) provided sufficient Cl^- was present in the preparations. This Ca^{2+} effect probably reflects binding of a single Ca^{2+} ion (Cammarata & Cheniae, 1987; Katoh et al., 1987). The loss of Ca^{2+} from NaCl-washed PS-II membranes is reported by some workers to be light dependent [Dekker et al., 1984; Miyao & Murata, 1986; see also Nakatani (1984)], although whether this is an absolute requirement is controversial [Miyao and Murata (1986) vs, e.g., Ghanotakis et al. (1984a-c); Ono & Inoue, 1986; de Paula et al., 1986; see also Discussion].

The step blocked under conditions of Ca^{2+} depletion is also controversial. Two different possibilities have been put for-

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[1] Abbreviations: P_{680} and P_{700}, reaction center chlorophyll (Chl) of photosystems II (PS-II) and I, respectively; Z, electron donor to P_{680}; Q_A, primary quinone electron acceptor of PS-II; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; cyt, cytochrome; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; Tris, tris(hydroxymethyl)aminomethane.

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ward: a block at the $S_1 \rightarrow S_2$ or at the $S_3 \rightarrow S_0$ transition. The conclusion that inhibition occurred at the S_1 to S_2 step was largely based on two kinds of EPR studies. First, by monitoring the yield of the EPR multiline signal from the S_2 state (Dismukes & Siderer, 1980), formed by illumination at 200 K in NaCl-washed membranes, Blough and Sauer (1984) and de Paula et al. (1986) [see also Miller et al. (1987)] found a decrease in the amplitude of the multiline signal relative to that observed in the reconstituted membranes with the 24-kDa protein or Ca^{2+}, respectively [see also Ghanotakis et al. (1987) but see, however, Tamura et al. (1986)]. Second, Cole and Sauer (1987) observed that the rate of Z^+ reduction was slower in NaCl-washed material and interpreted this as indicating a lack of electron donation from the S states to Z^+. Similar results were obtained by using repetitive flash and steady-state illumination methods (Ghanotakis et al., 1984a; Boska et al., 1985).

In contrast, studies of the luminescence emission that oscillates in phase with oxygen evolution (Zankel, 1971) and that occurs in the millisecond range in NaCl-washed membranes (Dekker et al., 1984) led to the conclusion that Ca^{2+} was essential for the $S_3Z^+ \rightarrow S_0$ transition (Boussac et al., 1985a). In agreement with this, Ono and Inoue (1986) found from thermoluminescence measurements that half of the inhibited centers in NaCl-washed PS-II membranes were blocked after S_3 formation. More recently it was shown, by luminescence measurements, that the charges stored in the absence of Ca^{2+} can be used in normal S-state cycling after rapid Ca^{2+} readdition (Boussac & Rutherford, 1988).

The existence of Ca^{2+}-binding sites with high (K_m = 50–100 μM) and low (K_7 = 1–2 mM) affinities in NaCl-washed PS-II membranes (Boussac et al., 1985b; Cammarata & Cheniae, 1987) is likely to be directly related to the discrepancies in the literature. The range of experimental protocols used by the many different groups has resulted in differential degrees of Ca^{2+} depletion from these binding sites. The most important experimental factors in this regard are the presence or the absence of light and chelating agents during or after the salt-washing procedure (see Table I).

In this work we present EPR data that support the view that the dominant effect of Ca^{2+} depletion is a block of oxygen evolution at the S_3 to S_0 transition. An attempt is made to reconcile these results with those already in the literature.

Studies of the specificity of the site for Ca^{2+} have shown that only Sr^{2+} gives significant reactivation of oxygen evolution under continuous illumination (Pistorius, 1983; Ghanotakis et al., 1984a). The partial reconstitution of activity by Sr^{2+} reported earlier was demonstrated to be due to a decrease of the quantum yield for oxygen evolution [Boussac & Rutherford, 1988; see also Volker et al. (1987)]. Here we report spectral changes in the S_2-multiline EPR signal due to replacement of Ca^{2+} by Sr^{2+}. Some of these results have been published in a preliminary form elsewhere (Boussac & Rutherford, 1988).

Experimental Procedures

Photosystem II particles from spinach chloroplasts 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 in 0.3 M sucrose, 30 mM NaCl, 25 mM MES, pH 6.5, and 30% (v/v) ethylene glycol at approximately 4 mg of Chl/mL. The activity of these particles varied from 400 to 450 μM O_2/(mg of Chl·h). NaCl washing of PS-II particles was done in room light at 4 °C at 0.5 mg of chlorophyll (Chl)/mL in 0.3 M sucrose, 1.2 M NaCl, and 25 mM MES, pH 6.5. After a 30-min incubation, 50 μM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added and the NaCl-washed particles were pelleted by 15-min centrifugation at 40000*g*, washed once in 30 mM NaCl, 25 mM MES, pH 6.5, and 50 μM EGTA, pelleted again, and resuspended at 8–12 mg of Chl/mL (3.5 mg of Chl/mL for flash experiment) in the same medium, in some cases with 20 mM $CaCl_2$ or $SrCl_2$. All these operations were done in room light. Sucrose was omitted to prevent possible Ca^{2+} contamination (Boussac et al., 1985b) and to allow faster Ca^{2+} reactivation (Boussac & Rutherford, 1988). The samples were put in calibrated quartz EPR tubes and, after 20 min of dark adaptation (10 min for flash experiments), frozen and stored at 77 K. For NaCl washing in the dark, the same protocol was used except that particles were incubated 45 min in 0.3 M sucrose, 2 M NaCl, 25 mM MES, pH 6.5, and 5 mM EGTA. After NaCl washing in the light, the residual oxygen evolution did not exceed 20–30% of the activity of the Ca^{2+}-reconstituted sample. The oxygen-evolving activity of the reconstituted sample was 80% that in the untreated membranes. In the experiment of Figure 5, the untreated particles were washed in 30 mM NaCl, 25 mM MES, pH 6.5, and 50 μM EGTA, pelleted, and resuspended in the same medium but with either 50 μM or 5 mM EGTA. $SrCl_2$ was obtained from Merck, and the $CaCl_2$ contamination did not exceed 0.0005%.

The samples were illuminated 5 min at 200 K in a solid CO_2–ethanol bath in a non-silvered Dewar by using an 800-W projector just before the recording of the spectra. A 2-cm path length of water and three Calflex filters were used to cut off infrared radiation. In flash experiments the samples were thawed before the addition of 50 mM $CaCl_2$ in some samples and 1 mM phenyl-*p*-benzoquinone (PPBQ), dissolved in dimethyl sulfoxide, as an electron acceptor. Flashes were provided from a Nd-YAG laser (15 ns, 300 mJ, 530 nm) at room temperature with a frequency of 1 Hz. The flash-illuminated samples were rapidly (less than 2 s) cooled to 200 K then to 77 K. After one flash no further signal could be induced by a 200 K illumination, confirming the saturating intensity of the flash. EPR spectra were recorded at liquid helium temperature with a Bruker ESR200D X-band EPR spectrometer equipped with an Oxford Instruments cryostat. A Tracor-Northern 1710 apparatus was used for averaging and subtraction of the spectra.

Results

Figure 1 shows the light-induced signals at 200 K in PS-II particles NaCl washed in the light (spectrum a) and after reconstitution with Ca^{2+} (spectrum b). Comparison of the amplitude of the two spectra shows that the S_2 state was produced in approximately the same yield with and without Ca^{2+} (see Table I). The shapes of the spectra are almost identical with each other. The amplitude of the multiline signal in NaCl-washed Ca^{2+}-reconstituted PS-II particles was approximately 80% that observed in untreated membranes [not shown, but see Boussac and Rutherford (1988) and also Blough and Sauer (1984) and de Paula et al. (1986)].

When PS-II membranes are illuminated by a flash sequence at room temperature, the amplitude of the multiline signal in the rapidly frozen samples oscillates with a period of four, characteristic of the charge storage mechanism of the oxygen-evolving complex and shows a maximum after the first flash when the concentration of the S_2 state is maximum (Dismukes & Siderer, 1980; Zimmermann & Rutherford, 1984). This can also be observed in NaCl-washed PS-II particles reconstituted by Ca^{2+} (Figure 2). Figure 3 shows typical traces after zero, one, three, and five flashes in the high

Table I: Comparison of S_2-State Formation and Oxygen Evolution Activity in NaCl-Washed and Reconstituted PS-II Membranes

references	species	VO_2^a (%)	VO_2^b (%)	observable	S_2^a (%)	S_2^b (%)	washing	notes
Blough and Sauer (1984)	spinach	22	30[c]	EPR multiline	50	60	light	no chelator
Boska et al. (1985)	spinach	31		Z^+ reduction by EPR	55		light	no chelator phases: 55%, 140 μs; 25%, 9 ms; 20%, 100 ms
Boussac et al. (1985a,b)	peas	10	15	luminescence	68	71	light	50 μM EGTA
Boussac and Rutherford (1988) and this work	spinach	16	20	EPR multiline	71	79	light	50 μM EGTA
Cole and Sauer (1987)	spinach	35		Z^+ reduction by EPR	40 + 30 ?		light	no chelator phases: 40%, <200 μs; 30%, 0.9–4.3 ms; 30%, 100 ms
de Paula et al. (1986)	spinach	28	39	EPR multiline	40	57	dark	5 mM EGTA
		34	48	EPR multiline	70	100	dark	no chelator
Ghanotakis et al. (1984a)	spinach	21		Z^+ reduction by EPR	50		dark	no chelator
Ono and Inoue (1985)	spinach	51		thermoluminescence	69		dark	no chelator
Ono and Inoue (1986)	spinach	51	71	thermoluminescence	75		dark	5 mM EDTA
	spinach	51	71	thermoluminescence	100		dark	no chelator
Radmer et al. (1986)	wheat	50	63	EPR multiline	100		light	no chelator
Tamura et al. (1986)	wheat	67	84[c]	TMPD oxidation	66	75	light	no chelator

[a] Oxygen evolution activity or amount or S_2 state after NaCl washing relative to untreated membranes. [b] Oxygen evolution activity or amount of S_2 state after NaCl washing relative to Ca^{2+}-reconstituted NaCl-washed membranes. [c] Reconstitution by the 24-kDa protein instead of Ca^{2+}.

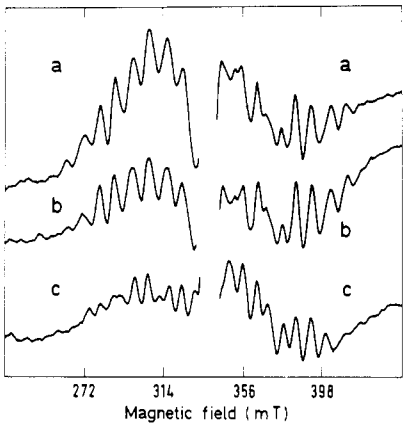

FIGURE 1: EPR difference spectra of PS-II particles NaCl washed in the light. The spectra are the difference between the spectra after 200 K illumination for 5 min and the spectra of the same dark-adapted sample: (a) nonreconstituted sample; (b) Ca^{2+}-reconstituted sample; (c) Sr^{2+}-reconstituted sample. Instrument settings: microwave frequency, 9.44 GHz; modulation amplitude, 22 G; temperature, 10 K; microwave power, 20 mW (10 dB). Each trace corresponds to the addition of 8 accumulations on 3 different samples (i.e., 24 accumulations). The right parts of the spectra have been drawn upwardly shifted.

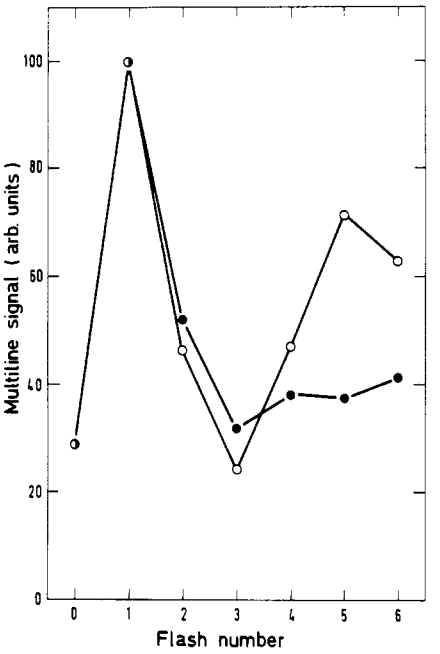

FIGURE 2: Amplitude changes of the multiline signal after a series of flashes at room temperature given on PS-II particles NaCl washed in the light. Nonreconstituted sample (●); Ca^{2+}-reconstituted sample (○). The amplitude of the multiline signal was estimated from the height of the three high-field peaks marked with an asterisk in Figure 3 and was normalized to the amplitude after one flash. Instrument settings were the same as in Figure 3. Each trace is the average of three different experiments.

magnetic field part of the spectra. It can be seen that the amplitude after one flash in the absence of Ca^{2+} (Figure 3A) represents 86% of the amplitude obtained in the presence of Ca^{2+} (Figure 3B). In the absence of Ca^{2+} the decrease in the amplitude of the multiline signal on the second flash (Figure 2) indicates that S_3 can be formed, while the lack of increase in the multiline signal on the fifth flash shows that an inhibition has occurred prior to this step. The higher level after two and three flashes in the absence of Ca^{2+} could be due to the shorter lifetime of the S_3 state (i.e., 4 s; Boussac et al., 1985a), which deactivates into the S_2 state between the flash and the freezing of the sample (1–2 s). The presence of some multiline signal prior to flash excitation is probably due to a shorter dark adaptation of the samples (approximately 10 min) used in these experiments to minimize any loss of activity in the salt-washed material. This, however, does not change the interpretation of the results, although the presence of some S_2 state in the dark-adapted sample explains why the amplitude of the signal in the presence of Ca^{2+} is smaller after three flashes than in the dark-adapted sample and also why there is a slight increase in the amplitude after four flashes. It should be noted that during the flash sequence, with or without Ca^{2+}, no change in the amplitude of signal II_{slow} occurred (not shown).

Figure 3 shows that when a sample lacking Ca^{2+} was frozen 15 s after the third flash, we observe a significant increase (from 25 to 72% of the signal after one flash) in the amplitude of the multiline signal. The formation of the S_2 state in the dark in this experiment is interpreted as being due to deactivation of the S_3 state and hence as indicating an inhibition of the S_3 to S_0 transition. In the sample reconstituted with Ca^{2+}, three flashes advance most of the centers to the S_0 state, and thus very little S_2 formation occurs (from 43 to 45% of the signal after one flash) upon subsequent dark period. The value of 15 s was long enough to ensure complete deactivation

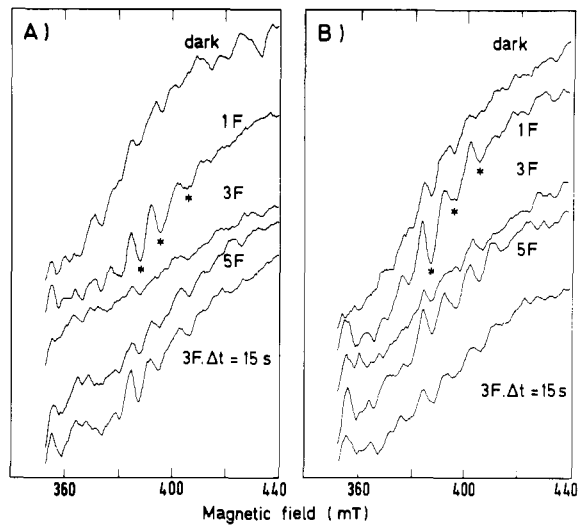

FIGURE 3: EPR spectra of PS-II membranes NaCl washed in the light, generated by a series of flashes given at room temperature. (A) Nonreconstituted sample; (B) Ca^{2+}-reconstituted sample. The time between the flash and the freezing was less than 2 s or equal to 15 s when indicated. Instrument settings: temperature, 8 K; modulation amplitude, 31 mW (8 dB); microwave frequency, 9.44 GHz; 8 accumulations. These data are from an experiment different form those in Figure 2.

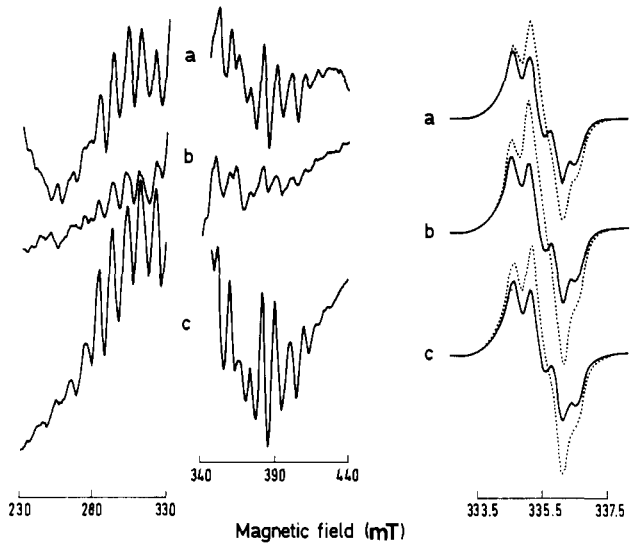

FIGURE 4: EPR spectra of PS-II NaCl washed in the dark with 5 mM EGTA resuspended in media containing (a) 50 μM EGTA, (b) 10 mM EGTA, or (c) 20 mM $CaCl_2$. The spectra are the difference between the spectra of the same dark-adapted sample. Instrument settings: microwave frequency, 9.44 GHz; modulation amplitude, 22 G; temperature, 10 K; microwave power, 20 mW (10 dB). Each trace corresponds to four accumulations. (Right) $g = 2$ region before (continuous line) or after (dashed line) 200 K illumination for 5 min. Instrument settings: microwave frequency, 9.44 GHz; modulation amplitude, 2.8 G; temperature, 15 K; microwave power, 0.2 μW (60 dB).

of the S_3 state and short enough to prevent deactivation of the S_2 state in the NaCl-washed membranes without Ca^{2+} (Boussac et al., 1985a).

Since it has been reported earlier that Ca^{2+} is required for S_2-multiline signal formation (de Paula et al., 1986), we considered the possibility that the inhibition of multiline signal formation on the fifth flash could be due to a flash-induced release of residual Ca^{2+} occuring after the first flash. This possibility can probably be eliminated on the basis of two observations. First, in the experiment described above, the formation of the S_2-multiline signal by deactivation of the S_3 state in the dark and in the presence of 50 μM EGTA indicates that no residual Ca^{2+} release occurred after three flashes. Second, an experiment was performed in which NaCl-washed membranes in the presence or absence of Ca^{2+} were submitted to an additional period of illumination at room temperature in the presence of 1 mM PPBQ (not shown). Such illumination is known to remove any residual Ca^{2+}. The amplitudes of the light minus dark spectra induced by 200 K illumination of NaCl-washed PS-II centers preilluminated and dark-adapted at room temperature were compared with samples $\pm Ca^{2+}$. With Ca^{2+} no change in the amplitude of the signal occurs. In the Ca^{2+}-deficient sample only a small decrease in amplitude is observed (approximately 20%). This decrease is probably due to some photoinhibition of the centers blocked after the S_3 state, as observed in PS-II centers inhibited on the donor side (Callahan et al., 1986; Theg et al., 1986). From these experiments it is concluded that the multiline spectrum can be formed in the absence of Ca^{2+}.

We have looked for possible reasons why a decrease in the multiline signal was previously reported under similar conditions (Blough & Sauer, 1984; de Paula et al., 1986; Ghanotakis et al., 1987). Using material NaCl washed in the dark and further treated with 5 mM EGTA, de Paula et al. (1986) concluded that Ca^{2+} depletion inhibited the S_1 to S_2 transition. We therefore performed experiments using membranes NaCl washed in the dark with 2 M NaCl and 5 mM EGTA. Then PS-II particles were resuspended in a medium containing either 50 μM or 10 mM EGTA or 20 mM $CaCl_2$. Figure 4 shows the light minus dark spectra induced by a 200 K illumination of these samples and the signals before and after illumination in the region of signal II. It can be seen that the multiline signal was significantly smaller with 10 mM EGTA.

The loss of the multiline signal was not accompanied by an increase in the $g = 4.1$ signal, a different form of the S_2 state (Zimmermann & Rutherford, 1986; Beck & Brudvig, 1986). Lack of S_2 formation at 200 K is usually accompanied by either oxidation of cyt b_{559} or oxidation of a chlorophyll (de Paula et al., 1985). After NaCl washing, however, cyt b_{559} is in its low-potential form and already oxidized even in the dark [not shown, but see Ghanotakis et al. (1986) and de Paula et al. (1986)]. In the three samples the illumination produced the same small amount of free radical at $g = 2.0026$ attributed to a chlorophyll cation. Some of this signal, however, may be due to a very slight P_{700}^+ contamination.

The spectrum recorded in the presence of 50 μM EGTA shows that the decrease in the multiline was largely reversible when the high concentration of EGTA was removed. This effect is probably not due to reconstitution by residual amounts of Ca^{2+} because 50 μM EGTA is still present in the medium.

Figure 5 shows the microwave saturation curves of the multiline signal in the presence of 50 μM or 5 mM EGTA in untreated (Figure 5B) and NaCl-washed (Figure 5A) PS-II membranes. The effect on the amplitude of the signal is more pronounced at low microwave power, and the shape of the curves differs at high power. The insets of Figure 5 show the microwave-saturating behavior of the multiline signal, with 50 μM and 5 mM EGTA, normalized to the same amplitude in the nonsaturating part of the curves. The amplitude of the multiline signal recorded under nonsaturating conditions in the presence of 5 mM EGTA represents approximately 60% that with 50 μM EGTA. These results indicate a direct effect of EGTA on the amplitude and relaxation properties of the S_2-multiline signal.

Illumination at 77 K allows observation of the EPR signals, at $g = 1.9$ and $g = 1.82$, without the overlapping S_2-multiline signal. These signals are due to the semiquinone–iron complex

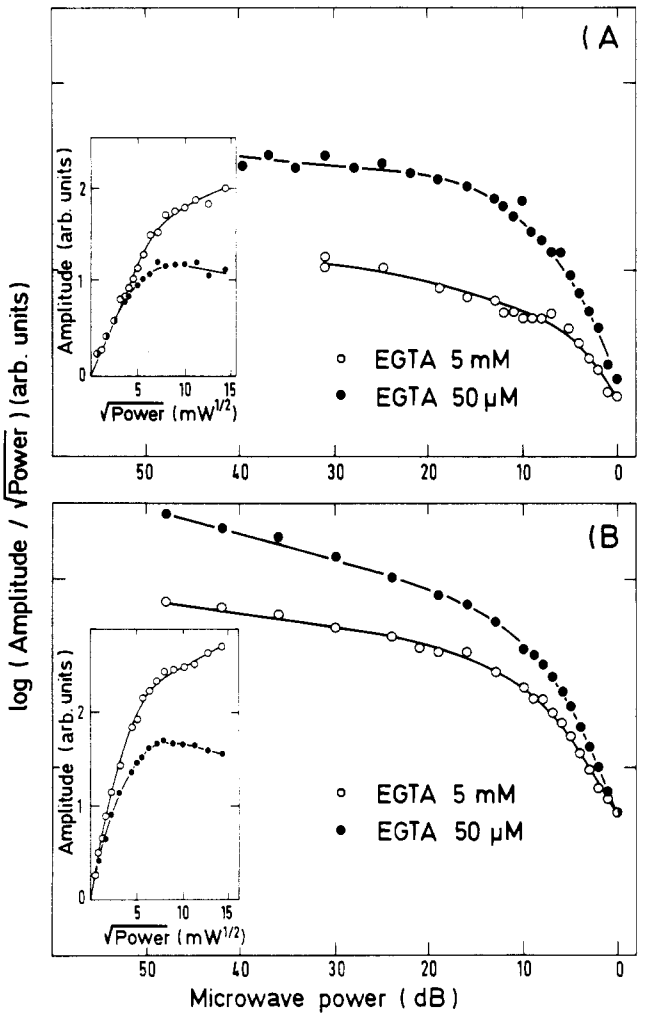

FIGURE 5: Saturation curves of the multiline signal in (A) NaCl-washed membranes in the dark in the presence of 5 mM EGTA or (B) untreated PS-II membranes resuspended in 50 μM (●) or 5 mM EGTA (○). The amplitude of the multiline signal was estimated from the height of the three high-field peaks marked with an asterisk in Figure 3. Instrument settings are the same as in Figure 4. The Chl concentration in (B) is 1.5 times higher than in (A). The insets show the amplitude of the multiline signal recorded in the presence of 50 μM or 5 mM EGTA and normalized to the same value under non-saturating conditions versus the square root of the microwave power.

$O_A^-Fe^{2+}$ (Rutherford & Zimmermann, 1984; Nugent et al., 1981). NaCl-washed samples exhibit principally the $g = 1.82$ form (not shown), and it seems unaffected by EGTA. The apparently larger signal at $g = 1.9$ in Figure 4a,c is due to a large underlying derivative-shaped spectrum that is associated with the S_2-multiline signal (Hansson et al., 1987). Note also that the right part of the spectra has been drawn upwardly shifted relative to the left part of the spectra. Thus, the decrease in the amplitude of the multiline signal is unlikely to be due to a decrease in the yield of stable photochemistry at low temperature. It should also be noted that oxygen evolution, under continuous light, by untreated PS-II membranes is the same in media containing 50 μM or 10 mM EGTA [not shown; see also Ghanotakis et al. (1984c)].

Sr^{2+} is known to specifically replace Ca^{2+} in its role in oxygen evolution (see the introduction). This replacement results in a modification of the S_2-multiline EPR spectrum (Figure 1, spectrum c). These modifications are more clearly demonstrated in Figure 6 when the spectra are recorded with a lower modulation amplitude. It can be seen that the reconstitution by Sr^{2+} (spectrum b) instead of Ca^{2+} (spectrum a) results in losses, shifts, splittings, and redistributions of the

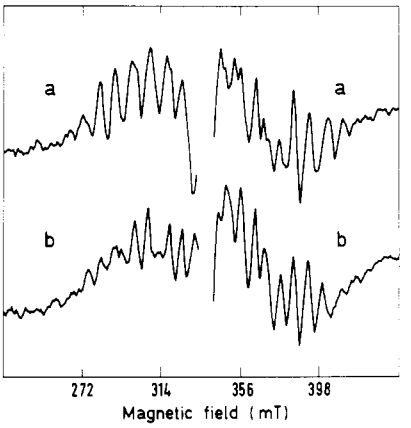

FIGURE 6: EPR difference spectra of PS-II particles NaCl washed in the light. The spectra are the difference between the spectra after 200 K illumination for 5 min and the spectra of the same dark-adapted sample: (a) Ca^{2+}-reconstituted sample; (b) Sr^{2+}-reconstituted sample. Instrument settings: microwave frequency, 9.44 GHz; modulation amplitude, 12.5 G; temperature, 10 K; microwave power, 20 mW (10 dB). Each trace corresponds to the addition of 8 accumulations on 3 different samples (i.e., 24 accumulations). The right parts of the spectra have been drawn upwardly shifted.

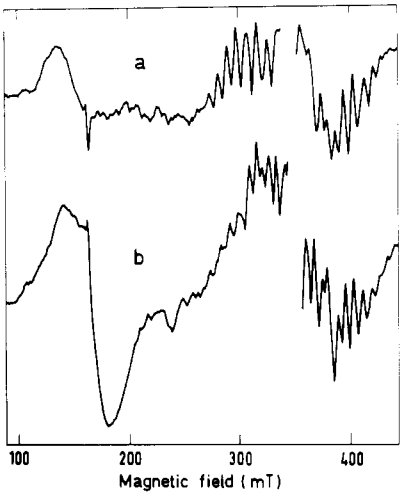

FIGURE 7: EPR difference spectra of PS-II particles NaCl washed in the light. The spectra are the difference between the spectra after 200 K illumination for 5 min and the spectra of the same dark-adapted sample: (a) Ca^{2+}-reconstituted sample; (b) Sr^{2+}-reconstituted sample. Instrument settings: microwave frequency, 9.44 GHz; modulation amplitude, 22 G; temperature, 10 K; microwave power, 20 mW (10 dB). Each trace corresponds to four accumulations. The right parts of the spectra have been drawn upwardly shifted.

amplitude of the different lines. The averages of the hyperfine line spacings are 87.9 and 71.0 G for Ca^{2+}- and Sr^{2+}-reconstituted samples, respectively. These values agree with those reported by Beck et al. (1986) for untreated (87.5 G) and NH_3-treated PS-II membranes (67.5 G). We have checked, by illumination of two similar samples at 77 K, that no modifications of the signal arising from $Q_A^-Fe^{2+}$ occur (not shown). A fraction of PS-II centers in untreated particles have an S_2 state at $g = 4.1$ instead of the multiline signal (Zimmermann & Rutherford, 1986). Very little of this signal can be induced after NaCl washing even after reconstitution by Ca^{2+} [Figure 7, spectrum a; see also de Paula et al. (1986)]. The light minus dark spectrum induced by a 200 K illumination shows that replacement of Ca^{2+} by Sr^{2+} results in the reappearance of a signal at $g = 4.1$ (Figure 7, spectrum b).

DISCUSSION

The results presented in this paper (Figures 1–3) show that, after NaCl washing of PS-II membranes in the light and

resuspension in the presence of 50 μM EGTA, oxygen evolution is inhibited at the S_3 to S_0 transition. This inhibition is overcome by the addition of Ca^{2+}. This conclusion agrees with some previous reports (Boussac et al., 1985a; Ono & Inoue, 1986; Boussac & Rutherford, 1988) but disagrees with the conclusion of several others groups [Blough & Sauer, 1984; Boska et al., 1985; de Paula et al., 1986, Cole & Sauer, 1987; Miller et al., 1987; Ghanotakis et al., 1987; see also Tamura et al. (1986)]. In the following discussion attempts are made to reconcile these conflicting reports with the present paper.

Some of the conflicting conclusions were based on low-temperature EPR measurements similar to those reported here. Although Blough and Sauer (1984) and de Paula et al. (1986) both observed a decrease in the S_2-multiline signal, it can be seen from Table I that this did not correspond closely to a much more significant loss of oxygen evolution. Indeed, when comparisons of the S_2-multiline signal amplitudes in depleted PS-II membranes are made relative to those in reconstituted membranes rather than to untreated membranes, approximately 60% of the S_2-multiline signal amplitude remains. These results do not clearly indicate that inhibition occurs solely prior to S_2 formation. Some experimental factors may also have contributed to the decrease in the multiline signal observed earlier. First, de Paula et al. (1986) included a high concentration of EGTA in the EPR samples. Here we have shown that EGTA itself can significantly diminish the S_2-multiline signal amplitude, and this effect is apparently unrelated to Ca^{2+} binding (Figures 4 and 5). Possible conformational effects around the Mn cluster due to chelating agents have been discussed earlier from NMR data (Wydrzynski & Renger, 1986). In addition, it is known that acetate acts as an inhibitory counterion for Cl^- in oxygen evolution (Critchley, 1985), and it is possible that the four acetate groups of EGTA could have an effect on the Mn cluster. Second, Blough and Sauer (1984) resuspended the NaCl-washed membranes in 10 mM Cl^-, a concentration that is suboptimal for oxygen evolution (Murata & Miyao, 1985). This could lead to a diminished multiline signal amplitude [see Ono et al. (1986)].

Miller et al. (1987) reported a clear reversible effect of Ca^{2+} on the multiline signal in the absence of EGTA. This work, however, differs from the majority of reports in that the PS-II membranes used also lacked the 33-kDa protein. Depletion of Ca^{2+} under these conditions could well result in a greater perturbation of the Mn cluster, resulting in loss of the multiline signal. Nevertheless, in the same kind of material, Ono and Inoue (1985) showed by thermoluminescence that S_2 could be formed to an amplitude that was 80% that in untreated membranes. These thermoluminescence measurements were done on membranes suspended in a buffer that contained no Ca^{2+} and only 10 mM Cl^-, and, on the first six flashes, oxygen evolution was decreased to 54% that in untreated membranes (Ono & Inoue, 1986).

A study of Z^+ reduction kinetics after a series of flashes in NaCl-washed PS-II membranes led Cole and Sauer (1987) to conclude that inhibition of S-state formation occurred in the absence of Ca^{2+}. However, their results showed that, first, in the majority of centers (approximately 40%) electron donation to Z^+ remained faster than the time response of the instrument. Second, in a fraction of the centers (approximately 30%) electron donation was slower than normal, but the kinetics were still dependent on the S state, and the half-time after the third flash corresponded to the $S_3 \rightarrow S_0$ transition measured in NaCl-washed PS-II membranes (Dekker et al., 1984). Third, in only a small fraction of the centers (approximately 15%) electron donation to Z^+ was reversibly inhibited, giving kinetics like those in Tris-washed material. Since Blough and Sauer (1984) had previously concluded that S_2 formation was inhibited in NaCl-washed material, Cole and Sauer (1987) interpreted their data in line with the earlier repetitive flash kinetic data of Boska et al. (1985), i.e., that S_2 formation was blocked but that another carrier Y, which was proposed to exist between Z and the S states, could still function. On the basis of the present work showing S_2 formation under similar conditions, it seems likely that the kinetics which had previously been attributed to electron donation from Y to Z^+ can be reattributed to modified donation kinetics from the S states when Ca^{2+} is depleted. In any case these kinetic data cannot be considered as strong evidence for inhibition of S-state advancement.

Tamura et al. (1986) used the rate of oxidation of TMPD, an exogenous electron donor, as a probe of the S_2 state in NaCl-washed PS-II membranes from wheat. A clear correlation between the loss of oxygen evolution and of the S_2 state was observed. This indicates an electron-transfer inhibition prior to S_2-state formation. However, 84% of oxygen evolution activity and 75% of S_2 state were present in the NaCl-washed material relative to those values after reconstitution with the 23-kDa protein. Cammarata and Cheniae (1987) later showed that the procedure used by Tamura et al. (1986) [and Radmer et al. (1986)] resulted in the complete depletion of Ca^{2+} from the low-affinity site, while the high-affinity remained unaffected. In agreement with earlier work (Boussac et al., 1985b), Cammarata and Cheniae (1987) also showed that the high-affinity site was depleted if, in addition to NaCl washing in room light, a treatment with EGTA was also given. From the studies of Boussac et al. (1985b) and Cammarata et al. (1987), it emerges that the high-affinity site was present in 70–75% of PS-II centers, while the remaining centers possess a low-affinity site. Under conditions in which the high-affinity site is depleted, the oxygen activity is much more severely inhibited (see Table I). It is this kind of treatment that is used in the present work and in earlier work (Boussac et al., 1985a,b; Boussac & Rutherford, 1988) and in which the major effect of Ca^{2+} depletion is an inhibition of the S_3 to S_0 transition. On this basis and from the work of Cammarata and Cheniae (1987) and Tamura et al. (1986), it appears that the complete depletion of Ca^{2+} from the low-affinity site correlates with an inhibition of S_2 formation in a small fraction of the centers, while the depletion of Ca^{2+} from the high-affinity site correlates with a block after S_3 in the majority of the centers. This correlation not only is open to experimental verification but also helps to explain the range of inhibitory effects observed in the literature.

Table I shows a collation of most of the pertinent literature on effect of Ca^{2+} depletion and reconstitution. Data where inhibition seems to be largely due to Cl^- depletion have been avoided [see Ono et al. (1986) and Styring et al. (1987) for discussions]. In nearly all of the cases in which the S_2 formation was estimated, the amount of the S_2 state remaining after NaCl washing was greater than the percentage of oxygen evolution activity in the same material. There are two likely reasons for this: First, when Ca^{2+} is only partially depleted (by NaCl washing in the light but no chelators or in the dark plus chelators), then the continuous illumination used for the measurement of oxygen evolution results in further Ca^{2+} depletion [see Natakani (1984) and Dekker et al. (1984)]. Thus, the results of oxygen evolution measurements in salt-washed material may depend strongly on just how the initial rate is measured. Second, when Ca^{2+} is extensively depleted (by NaCl washing in the light with chelators), the inhibition is

dominated by a block after S_3 formation; thus, S_2 can be formed (this work; Boussac et al., 1985a).

The significance of the apparent heterogeneity of PS-II in which a fraction (25%) of the centers have a low-affinity Ca^{2+}-binding site and the majority (75%) of the centers have a high-affinity Ca^{2+}-binding site (Boussac et al., 1985b; Cammarata & Cheniae, 1987) is at present unclear. However, it is tempting to equate these numbers to the dark distribution of the S states (Kok et al., 1970). S-state dependent Ca^{2+} affinities could at least partially explain the role of light in Ca^{2+} depletion protocols. In other words, we would predict that all centers could potentially exhibit both kinds of Ca^{2+}-binding affinity. Thus, the Ca^{2+} specific binding affinities observed for photoactivation (approximately 2 mM) (Ono & Inoue, 1983b; Tamura & Cheniae, 1987), the H_2O_2-driven S_1/S_{-1} cycling (1–2 mM) (Frash & Mei, 1987), and also perhaps Z^+ reduction in Tris-washed chloroplasts (2 mM) (Yerkes & Babcock, 1981) could all be different manifestations of the same low-affinity Ca^{2+}-binding site characterized in NaCl-washed PS-II membranes [Boussac et al., 1985b; Cammarata & Cheniae, 1987; see also Ghanotakis et al. (1984c) and Packham and Barber (1984)]. It is more than coincidental that in all of these studies the 18- and 24-kDa proteins are either lacking or not bound to the membrane. Other effects of divalent cations have been reported on the donor side of PS-II, which probably reflect nonspecific surface charge phenomena (Yerkes & Babcock, 1981).

In this paper we have also presented EPR spectra of S_2 in NaCl-washed PS-II membranes reconstituted with Sr^{2+} instead of Ca^{2+}. Sr^{2+} replacement has two effects. First, the multiline signal is modified in terms of its intensity, the number of peaks, and their splittings (Figures 1 and 6). Second, a significant increase in the amplitude $g = 4.1$ signal is induced. Very similar phenomena were induced when NH_3 was bound at two different sites (Beck et al., 1986; Beck & Brudvig, 1986; Andreasson & Hansson, 1987). In particular, the modifications of the multiline signal induced by Sr^{2+} are strikingly similar to those induced by NH_3 binding (Beck et al., 1986; Andreasson & Hansson, 1987; Aasa et al., 1987). This effect was previously considered as evidence that NH_3 binds directly to the Mn cluster in the substrate site. The observation that Sr^{2+} has a very similar effect casts doubt on this line of evidence. Both NH_3 and Sr^{2+} have an effect on the Mn cluster, not necessarily at the same site, but in such a way that it adopts the same specific conformation.

The replacement of Ca^{2+} by Sr^{2+} has been shown to give 40% oxygen evolution activity in saturating light intensity (Ghanotakis et al., 1984a), while in limiting light intensity 85% of the activity was observed (Boussac & Rutherford, 1988). This indicates a slowdown in the rate-limiting step in Sr^{2+}-reconstituted material. Since the rate-limiting step in untreated (approximately 1 ms) and in NaCl-washed (approximately 4 ms) (Dekker et al., 1984) PS-II membranes is at the S_3 to S_0 transition and since it is at this step that inhibition occurs when Ca^{2+} is depleted, it seems reasonable to assume that Sr^{2+} binding gives a slower rate for the S_3 to S_0 transition and hence a greater miss factor at this step.

An alternative explanation for this effect should, however, be considered. Beck and Brudvig (1988) recently proposed that the multiline and $g = 4.1$ forms of the S_2 state are in equilibrium at room temperature and that the $g = 4.1$ form is unable to advance to the S_3 state. The equilibrium distribution of the two forms, in their model, would determine the miss factor on the S_2 to S_3 transition. The observation that reconstitution of Ca^{2+}-depleted PS-II membranes with Sr^{2+} enhances the $g = 4.1$ form could at least partially explain the lower rate of oxygen evolution. These alternative explanations are clearly testable.

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Evidence That the Two Amino Termini of Plasma Fibronectin Are in Close Proximity: A Fluorescence Energy Transfer Study†

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ABSTRACT: A fluorescence energy transfer technique has been used to study the intramolecular distance between the two amino termini of human plasma fibronectin. The glutamine-3 residue near the amino terminus of each chain was labeled enzymatically with either monodansylcadaverine or monofluoresceinylcadaverine by use of coagulation factor XIIIa. The nonradiative fluorescence energy transfer between the dansyl (donor) and fluorescein (acceptor) pair in the same protein molecule was determined from steady-state fluorescence measurements. On the basis of the transfer efficiency of 78%, the intramolecular distance between two glutamine-3 residues of fibronectin was estimated to be approximately 23 Å, suggesting that the two amino termini of plasma fibronectin are in close proximity. High salt, which affects the hydrodynamic properties of the protein, has no effect on the measured distance. The results support the contention that both compact (in low salt) and expanded (in high salt) conformers of fibronectin are relatively spherical in shape.

Plasma fibronectin (Fn) is a glycoprotein, consisting of two subunits of about 250 kDa each which are joined at their carboxyl termini by two disulfides [for reviews, see McDonagh (1985) and Yamada (1983)]. The protein is known to participate in blood coagulation, cell adhesion, differentiation, and embryonic development.

The solution structure of this multifunctional, multidomainal

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