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Inhibition of Tyrosine Z Photooxidation after Formation of the S_3 State in Ca^{2+}-Depleted and Cl^--Depleted Photosystem II

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ABSTRACT: Ca^{2+} and Cl^- are obligatory cofactors in photosystem II (PS-II), the oxygen-evolving enzyme of plants. The sites of inhibition in both Ca^{2+}- and Cl^--depleted PS-II were compared using EPR and flash absorption spectroscopies to follow the extent of the photooxidation of the redox-active tyrosine (TyrZ) and of the primary electron donor chlorophyll (P_{680}) and their subsequent reduction in the dark. The inhibition occurred after formation of the S_3 state in Ca^{2+}-depleted PS-II. In Cl^--depleted photosystem II, the inhibition occurred after formation of the S_3 state in about half of the centers and probably after S_2TyrZ^+ formation in the remaining centers. After the S_3 state was formed in Ca^{2+}- and Cl^--depleted photosystem II, electron transfer from TyrZ to P_{680} was inhibited. This inhibition is discussed in terms of electrostatic constraints resulting from S_3 formation in the absence of Ca^{2+} and Cl^-.

Photosystem II (PS-II)[1] catalyzes light-driven water oxidation resulting in oxygen evolution. The reaction center of PS-II is made up of two membrane-spanning polypeptides (D1 and D2) analogous to the L and M subunits of the purple photosynthetic bacteria [see Michel and Deisenhofer (1988) for a review]. Absorption of a photon leads to a charge separation between a chlorophyll molecule, designated P_{680}, and a pheophytin molecule. The pheophytin anion transfers the electron to a quinone, Q_A, and P_{680}^+ is reduced by a tyrosine residue, TyrZ, tyrosine-161 of the D1 polypeptide (Barry & Babcock, 1987; Debus et al., 1988a,b; Vermaas et al., 1988; Metz et al., 1989). A cluster of four Mn located in the reaction center of PS-II probably acts both as the active site and as a charge-accumulating device of the water-splitting enzyme [see Rutherford (1989) for a review]. During the enzyme cycle, the oxidizing side of PS-II goes through five different redox states that are denoted S_n, n varying from 0 to 4 (Kok et al., 1970). In oxygen-evolving PS-II, reduction of P_{680}^+ by TyrZ, after a flash, occurs in the tens to hundreds of nanoseconds time range depending on the S state (Brettel et al., 1984).

Three extrinsic polypeptides, with molecular masses of 17, 23, and 33 kDa, are bound to the PS-II reaction center on the inside of the thylakoid membrane. Removal of these three polypeptides by Tris washing results in the depletion of the Mn cluster and in inhibition of oxygen evolution [reviewed in Murata and Miyao (1985)]. In Tris-washed PS-II, reduction of P_{680}^+ by TyrZ occurs in about 5 μs at high pH and in 40-50 μs at low pH (Conjeaud & Mathis, 1980). This pH effect was interpreted as being due to protonation of a group near TyrZ (Conjeaud & Mathis, 1986). When TyrZ is already oxidized by a preflash, the charge recombination between P_{680}^+ and Q_A^- takes 200 μs (Conjeaud & Mathis, 1980). When the donation by TyrZ to P_{680}^+ is inhibited in the presence of acetate, the charge recombination between P_{680}^+ and Q_A^- takes 500 μs (Saygin et al., 1986). In a mutant of Synechocystis 6803 in which tyrosine-161 of the D1 polypeptide was replaced by a phenylalanine residue, the charge recombination between P_{680}^+ and Q_A^- occurs with a $t_{1/2}$ of 1 ms (Metz et al., 1989). Recently, Hogansson et al. (1991) reported that, in Tris-washed PS-II, the binding of Mn^{2+}, in a site probably corresponding to a Mn site in the functional enzyme, slowed down P_{680}^+ reduction by TyrZ from $\approx$5 to $\approx$40 μs. The variations in P_{680}^+ reduction kinetics described above were generally interpreted as being due to electrostatic restraints (Brettel et al., 1984; Hogansson et al., 1991; Metz et al., 1989).

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[1] Abbreviations: P_{680}, reaction center chlorophyll (Chl) of photosystem II (PS-II); TyrZ, tyrosine acting as the electron donor to P_{680}; TyrD, tyrosine acting as a side-path electron donor of PS-II; Q_A, primary quinone electron acceptor of PS-II; Q_B, secondary quinone electron acceptor of PS-II; EPR, electron paramagnetic resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PPBQ, phenyl-*p*-benzoquinone.

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The 17- and 23-kDa extrinsic polypeptides can be removed by NaCl washing [reviewed by Murata and Miyao (1985)]. Removal of these polypeptides results in inhibition of oxygen evolution due to an increased requirement for chloride and calcium ions [reviewed in Murata and Miyao (1985), Homann (1987), Debus (1991), and Yocum (1991)].

In Ca^{2+}-depleted PS-II, inhibition of the enzyme cycle occurs at the S_3 to S_0 transition (Boussac, et al., 1985b; Boussac & Rutherford, 1988a). This conclusion, however, has been controversial. Detailed rationalizations of conflicting views can be found in Boussac and Rutherford (1988a), Boussac et al. (1990b), and Rutherford et al. (1992).

We recently developed a salt washing procedure in which PS-II membranes were first depleted of Ca^{2+} and of the 17- and 23-kDa polypeptides in the presence of high concentrations of EGTA and light and then both polypeptides were reconstituted (Boussac et al., 1989). This preparation was incapable of O_2 evolution until Ca^{2+} was added. An EPR study of the inhibited system revealed the presence of a modified S_2 multiline signal which arises from a stable S_2 state (Boussac et al., 1989). Similar modified S_2 states were observed by other groups using different procedures (Sivaraja et al., 1989; Ono & Inoue, 1990). We subsequently demonstrated that the exposure of the Ca^{2+}-depleted enzyme to chelators or citrate [both poly(carboxylic acids)] in the S_3 state was required to induce the modifications (Boussac et al., 1990b).

Formation of the S_3 state in Ca^{2+}-depleted PS-II results in the appearance of an EPR signal centered at $g = 2$ with a line width of 130–164 G in the absence or the presence of the 17- and 23-kDa polypeptides, respectively (Boussac et al., 1989, 1990b). This signal was attributed to an organic free radical interacting magnetically with the Mn cluster (Boussac et al., 1989, 1990a) and, from its UV spectrum, was attributed to an oxidized histidine residue (Boussac et al., 1990a).

The depletion of Cl^- from PS-II also results in inhibition of oxygen evolution [reviewed in Coleman (1990), Homann (1987), and Rutherford et al. (1992)]. Two different types of inhibition have been observed, depending on the nature of the counterion used and/or the severity of the Cl^- depletion. A decrease in oxygen evolution attributed to a slow down of the S_3 to S_0 transition was reported when nitrate or acetate were used as counterions (Sinclair, 1984). In contrast, a total block in the S-state cycle was observed when sulfate was used as a counterion (Sinclair, 1984). The point at which the enzyme cycle is blocked by this kind of Cl^- depletion procedure has been investigated using a range of techniques, but some contradictory interpretations and observations have been reported. From luminescence and fluorescence studies, it was suggested that electron transfer was blocked after formation of the S_2TyrZ^+ state (Itoh et al., 1984; Theg et al., 1984). This was supported by using low-temperature EPR (Ono et al., 1986a), by flash absorption spectroscopy (Ono et al., 1986b), and by thermoluminescence (Vass et al., 1987). Earlier thermoluminescence studies, however, had been interpreted as indicating that inhibition occurred after S_3 formation (Homann et al., 1986). In another thermoluminescence study, Inoue (1987) observed a block after S_2TyrZ^+ formation in a part of the centers and after S_3 formation in the remaining centers.

Recently, Baumgarten et al. (1990) reported an EPR study of PS-II membranes which were depleted of Cl^- using F^- as a counterion, and an EPR signal was observed after flash or continuous illumination which bore some resemblance to the S_3 signal characterized earlier in Ca^{2+}-depleted material (Boussac et al., 1989). This result indicated that the roles of Ca^{2+} and Cl^- were interrelated. Such an interrelationship had been suggested earlier (Coleman, 1990; Homann, 1987), and a model in which these cofactors control the access of water to the active site has been put forward (Rutherford, 1989). In addition, experimental evidence for the sequential binding of Ca^{2+} and Cl^- has been reported (Waggoner & Yocum, 1990).

In the present paper, we report the further characterization of the electron-transfer reactions occurring in PS-II inhibited by Ca^{2+} depletion. During the course of the study done to obtain the UV spectrum of S_3 (Boussac et al., 1990a), no flash-induced absorption changes characteristic of $TyrZ^+$ could be observed after the formation of S_3 (Lavergne and Boussac, unpublished results). Therefore, here we have used kinetic methods to monitor $TyrZ^+$ and P_{680}^+ at room temperature. Similar studies of PS-II inhibited by Cl^- depletion are presented along with some low-temperature EPR studies. A comparison of the two kinds of inhibition is made, and it is concluded that, in both cases, electron transfer is blocked between TyrZ and P_{680}^+ after formation of S_3.

Materials and Methods

Photosystem II membranes 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 until used. The activity of these membranes was ≈600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. "NaCl-washed" membranes were prepared as in Boussac and Rutherford (1988a). "NaCl–EGTA-treated/polypeptide-reconstituted" samples were prepared as in Boussac et al. (1989). For flash absorption changes and EPR studies "NaCl–EGTA-treated/polypeptide-reconstituted" samples were resuspended in 0.3 M sucrose, 10 mM NaCl, and 25 mM Mes, pH 6.5. This medium was previously passed through a Chelex column to remove Ca^{2+} contamination. "NaCl-washed" membranes were used in 30 mM NaCl, 25 mM Mes, pH 6.5, and 50 μM EGTA. For EPR experiments, Cl^- depletion was done by two washings and resuspension of PS-II membranes in 0.3 M sucrose, 50 mM Na_2SO_4, and 50 mM HEPES, pH 7.5, as in Ono et al. (1986a). For flash absorption change studies, Cl^--depleted PS-II membranes were prepared by washing the membranes 3 times in 0.3 M sucrose–25 mM Mes, pH 6.5, leading to a residual Cl^- concentration less than 20 μM. Then the sample was stored at 0 °C in total darkness during the course of the experiment (1–6 hours with no significant decrease in oxygen evolution activity after reconstitution with Cl^-). Then, the sample (≈100 μL) was diluted into a cuvette of 2300 μL containing one of the following resuspension media: (1) a medium with 0.3 M sucrose, 50 mM Na_2SO_4, 10 mM $Ca(OH)_2$, and 50 mM HEPES, pH 7.5 [the pH was adjusted with NaOH after the addition of $Ca(OH)_2$]; (2) the same medium as above without $Ca(OH)_2$ but with 25 μM EGTA and passed through a Chelex column to remove Ca^{2+} contamination before the addition of EGTA. In low-temperature EPR experiments, 0.5 mM PPBQ, dissolved in dimethyl sulfoxide, was used as an electron acceptor whereas in all other experiments 1 mM ferricyanide was used.

For low-temperature EPR measurements, the samples were put into calibrated quartz tubes at a Chl concentration about 3 mg/mL, and after 30-min dark-adaptation on ice, PPBQ was added. EPR spectra were recorded at liquid helium temperatures with a Bruker ESR 200 X-band spectrometer equipped with an Oxford Instruments cryostat. The samples were either (1) illuminated in a nonsilvered Dewar flask in a solid CO_2–ethanol bath at 198 K or in an ethanol bath cooled to 0 °C with liquid nitrogen or (2) illuminated at room tem-

perature with flashes provided from a Nd–YAG laser (15 ns, 300 mJ, 530 nm). The flash-illuminated samples were rapidly (less than 2 s) cooled to 198 K and then to 77 K. For room temperature EPR measurements, the samples were put in a flat cell at a Chl concentration of 1.5 mg/mL. Illumination at room temperature was done with a saturating xenon flash or with continuous light. It was checked that no modification of the signal occurred during the accumulation.

Oxygen evolution in continuous saturating white light was measured by using a Clark-type electrode at 20 °C. Samples were diluted to 20 μg of Chl/mL in the indicated media in the presence of 1 mM PPBQ.

Flash-induced absorption experiments at 820 nm were done using a suspension of PS-II particles (100 μg of Chl/mL) in a square cuvette (10 × 10 mm). The measuring beam, provided by a tungsten–halogen lamp, was monochromated by interference filters before and after the cuvette. Excitation was provided by a Nd–YAG-pumped dye laser (broadband emission around 600 nm, pulse energy ≈ 15 mJ, duration 15 ns). The repetition rate was 1 Hz, except in those experiments in which the delay time was varied between the second and the third flash of a series. For measurements using a variable delay between the second and the third flash, the second flash was a xenon flash (5-μs duration). All flashes were saturating. A silicium photodiode was used as detector, and the output signal was filtered (10 Hz–1 MHz) and amplified before being recorded and digitized (RTD710A from Tektronix). A Marquardt least-squares algorithm was used to fit the relaxation kinetics to a multiexponential decay. The extinction coefficient for P_{680}^+ at 820 nm was taken as 7000 M^{-1} cm^{-1} (Mathis & Setif, 1981). The concentration of PS-II centers was estimated by taking 220 Chl/PS-II since we generally found 220 Chl/4 Mn by atomic absorption measurements (unpublished results). Due to the variation of the flattening effect with differently treated PS-II membranes in different media, the apparent extinction coefficient of P_{680}^+ at 820 nm may vary by 10–20% (Velthuys, 1988). Therefore, comparison between samples must take this uncertainty into account.

RESULTS

Ca^{2+} Depletion

Kinetic EPR Measurements of $TyrZ^+$. The EPR signal arising from photooxidized $TyrZ^+$ and its subsequent reduction in the dark was measured in a repetitive flash experiment with ferricyanide as an electron acceptor (Figure 1). Due to the time resolution of the EPR apparatus (time constant fixed to 1 ms), only the $TyrZ^+$ decaying in 1 ms or slower can be detected. Traces b–d were recorded in NaCl–EGTA-treated/polypeptide-reconstituted samples (Boussac et al., 1989), and to calibrate the amplitude of $TyrZ^+$, the signal was also recorded in a Tris-washed sample (trace a). It is known that, in Tris-washed PS-II, $TyrZ^+$ is reduced in the hundreds of milliseconds time range, allowing the EPR detection of one $TyrZ^+$ per PS-II center after a saturating flash [reviewed in Babcock (1987)]. $TyrZ^+$ is mainly reduced by the ferrocyanide produced by the reoxidation of Q_B^- and Q_A^- by ferricyanide and in a fraction of the centers by a pH-dependent charge recombination with the electron stored on the acceptor side (Dekker et al., 1984a). Reduction of $TyrZ^+$, in Tris-washed PS-II, is accelerated by cations (Yerkes & Babcock, 1981). In our conditions, in the presence of ferricyanide and Ca^{2+}, the $TyrZ^+$ in Tris-washed samples decays with a $t_{1/2}$ of 140 ms (trace a).

In oxygen-evolving PS-II, the electron reducing $TyrZ^+$ comes from the charge storage system associated with the oxidation of water. Of the four S-state transitions, only the S_3TyrZ^+ to S_0TyrZ transition is slow enough to be detected under our conditions (Blankenship et al., 1975; Babcock et al., 1976). The $t_{1/2}$ of this transition is 1 ms in untreated PS-II and irreversibly increases to 5 ms in salt-washed PS-II reconstituted with Ca^{2+} (Dekker et al., 1984b). In repetitive flash experiments, a mixing of the four S-state transitions occurs after a few flashes. Therefore, on a per flash basis, we expect to detect $TyrZ^+$ in only the 25% of PS-II centers in which the S_3TyrZ^+ to S_0TyrZ transition occurs. Trace b in Figure 1 shows $TyrZ^+$ formation and reduction in the NaCl–EGTA-treated/polypeptide-reconstituted sample incubated with 10 mM Ca^{2+}. This preparation exhibits high oxygen evolution activity [400–500 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}] in the presence of Ca^{2+} in the medium and no activity in the absence of Ca^{2+} [see also Boussac et al. (1989)]. The reduction of $TyrZ^+$ shown in trace b includes a small slow phase (15–20% of centers) corresponding to $TyrZ^+$ decaying with a $t_{1/2} \approx$ 100–200 ms. This slow phase probably corresponds to damaged PS-II centers which behave like Tris-washed PS-II. This assignment is supported by the fact that in a small proportion of centers, oxygen evolution is irreversibly inhibited. Nevertheless, as expected in functional PS-II, it can be seen that the amount of the fast-decaying $TyrZ^+$ ($t_{1/2} \approx$ 7 ms) in trace b of Figure 1 corresponds to approximately 25% of the amount of $TyrZ^+$ detected in Tris-washed samples.

Addition of Sr^{2+} instead of Ca^{2+} only partially reactivates oxygen evolution, and it was previously shown that this is due to a slowdown of the limiting rate (Boussac & Rutherford,

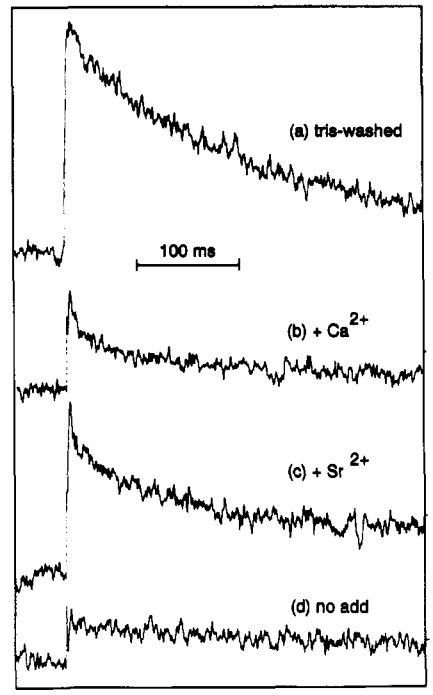

FIGURE 1: Reduction kinetics of $TyrZ^+$ in Ca^{2+}-depleted samples. Amplitudes of the EPR signal arising from $TyrZ^+$ induced by flash illumination and kinetics of the subsequent dark-reduction in a Tris-washed PS-II sample (trace a) or in NaCl–EGTA-treated/polypeptide-reconstituted samples (traces b–d). PS-II membranes were suspended in Chelex-treated medium containing 0.3 M sucrose, 10 mM NaCl, and 25 mM Mes, pH 6.5. For traces a and b, 10 mM $CaCl_2$ was added, and for trace c, 10 mM $SrCl_2$ was added. Each trace is the average of 1024 scans (8 samples and 128 scans per sample). The flash spacing was 4 s, and the signal was recorded during 400 ms. Instrument settings: temperature, ≈20 °C; microwave frequency, 9.85 GHz; modulation amplitude, 6.3 G; time constant, 1.25 ms; microwave power; 20 mW; chlorophyll concentration, 1.5 mg/mL. The traces were recorded at a magnetic field close to the low-field maximum of $TyrZ^+$.

Table I: Percentages of the Indicated Phases of P_{680}^+ Reduction Kinetics[a]

sample	$t_{1/2}$	flash no. 1	4	8	8 (+Ca^{2+})	8 (+Sr^{2+})
NaCl–EDTA-treated + polypeptides	10 μs	10 (10)	11 (11)	9 (9)	11 (34)	9 (19)
	20–40 μs	37 (37)	16 (16)	20 (20)	13 (40)	17 (38)
	150–200 μs	48 (48)	53 (53)	61 (61)	5 (14)	15 (33)
	1 ms	5 (5)	20 (20)	10 (10)	4 (11)	4 (9)
NaCl-washed	10 μs	8 (17)	8 (11)	5 (7)	7 (26)	
	20–40 μs	18 (36)	15 (23)	15 (19)	9 (36)	
	150–200 μs	17 (35)	36 (57)	49 (64)	6 (25)	
	≈1 ms	5 (11)	6 (9)	8 (19)	4 (14)	

sample	$t_{1/2}$	flash no. 2	3	8	8 (+Cl^-)
Cl^--depleted – Ca^{2+}	60–70 μs	17 (78)	38 (85)	67 (88)	74 (88)
	≈1 ms	5 (21)	7 (15)	10 (12)	10 (12)

[a] The first number indicates the absolute amplitude of each phase (1 P_{680}^+/PS-II is equal to 100%) and the number in parentheses the relative amount of the given phase on each flash. The extinction coefficient for P_{680}^+ is taken as 7000 M^{-1} cm^{-1}. The number of Chl per PS-II is estimated as 220.

1988b). Taking into account the minor slow phase which is attributed to damaged centers already seen in trace b, trace c of Figure 1 shows two effects induced by Sr^{2+} addition when compared to the signal in the presence of Ca^{2+}. First, $TyrZ^+$ reduction slowed down ($t_{1/2} \approx$ 15–20 ms), and second, the amplitude of the signal increased. This suggests that, in addition to the S_3TyrZ^+ to S_0TyrZ transition, the rate of $TyrZ^+$ reduction in another S-state transition was sufficiently slowed down to allow its detection.

In the absence of Ca^{2+} or Sr^{2+}, NaCl–EGTA-treated/polypeptide-reconstituted samples do not evolve oxygen at all (Boussac et al., 1989). Under these circumstances, the S-state cycle is inhibited after the formation of S_3 (Boussac et al., 1989). Nevertheless, trace d in Figure 1 shows that no $TyrZ^+$ was detected in repetitive flash experiments done in the absence of Ca^{2+} except for the small slow phase which is attributed to damaged centers. Very similar results were obtained in a different type of Ca^{2+}-depleted PS-II membranes, i.e., in NaCl-washed PS-II membranes which had not been treated with EGTA and had not been reconstituted with the 17- and 23-kDa polypeptides (not shown). Essentially the same results were also obtained when the EPR spectrum was detected under continuous illumination (not shown). In some Ca^{2+}-depleted preparations of PS-II, the amount of $TyrZ^+$ detected under continuous illumination was slightly larger [≈30% of the total amount of $TyrZ^+$, a value already observed (de Paula et al., 1986)] than the amount of $TyrZ^+$ detected in trace d of Figure 1. Nevertheless, this result rules out the possibility that $TyrZ^+$ was formed in Ca^{2+}-depleted PS-II centers but was missed in the kinetic experiments using a flash spacing of 4 s (Figure 1) because of an abnormally long lifetime.

As mentioned above, $TyrZ^+$ is not detectable in the millisecond time range in the functional enzyme because it is rapidly rereduced by the S states. In Ca^{2+}-depleted PS-II, it is known that the S-state cycle is blocked after S_3 formation (Boussac & Rutherford, 1988a); thus, it is unlikely that submillisecond rereduction of $TyrZ^+$ can explain the absence of the $TyrZ^+$ EPR signal. Much more likely is that TyrZ is not oxidized after formation of S_3. The formation of $TyrZ^+$ and its reduction by the S states on the first few flashes, even if kinetically detectable, would not have been detected since a large number of flashes were averaged.

Flash-Induced Absorption Studies of P_{680}^+. To obtain further information on the electron-transfer lesion in Ca^{2+}-depleted material, we measured P_{680}^+ reduction kinetics by flash-induced absorption changes in the microsecond time range. In general, P_{680}^+ reduction occurs in the submicrosecond time range in O_2-evolving PS-II and in the microsecond time range in inhibited PS-II. Monitoring P_{680}^+ reduction kinetics should allow us to determine whether electron donation from TyrZ occurs in Ca^{2+}-depleted PS-II. Figure 2 shows P_{680}^+ reduction kinetics after each flash (the number of which is indicated above the trace) of a sequence given to a dark-adapted sample. After the fourth flash, the signal was averaged over the next eight flashes; then, as indicated, Ca^{2+}, Sr^{2+}, or Mg^{2+} was added, and the signal was again averaged over eight flashes. This monitors the extent of reactivation of the submicrosecond P_{680}^+ reduction by these cations. Row a of Figure 2 corresponds to a NaCl–EGTA-treated/polypeptide-reconstituted sample. Unexpectedly, the first flash given to a dark-adapted sample results in P_{680}^+ which decays in the microsecond range. The amplitudes of the signals, induced by each flash, and their reduction kinetics were analyzed, and the more relevant results are presented in Table I. In all cases, the best fit was obtained with $t_{1/2}$ values of 10-μs, 20–40-μs, 150–200-μs, and 1-ms time ranges. The amplitudes of the signals in row a did not vary significantly from the first to the fourth flash (only the first and fourth flashes of the sequences are shown in row a). The signal averaged over the following eight flashes did not show any increase and corresponds approximately to one P_{680}^+ detected per PS-II center. Addition of Ca^{2+} or Sr^{2+} resulted in reactivation of electron donation to P_{680}^+ in the submicrosecond range. In contrast, Mg^{2+} was unable to reconstitute submicrosecond reduction of P_{680}^+. The effect of these added divalent cations corresponds to their ability to reactivate oxygen evolution in this preparation (Boussac et al., 1989). It is of note that trypsin treatment of PS-II has been reported to inhibit oxygen evolution similarly to Ca^{2+} depletion (Völker et al., 1987). These authors have studied the microsecond reduction kinetics of P_{680}^+ in this material in the absence or the presence of Ca^{2+}, Sr^{2+}, or Mg^{2+}. The results of Figure 2 are very similar to those of Völker et al. (1987).

From Figure 2, it can be deduced that no P_{680}^+ reduction occurred in the nanosecond time range in the absence of Ca^{2+} and that the kinetics were not markedly dependent on flash number. However, from Table I, some significant changes in the kinetics can be observed. From the EPR work in Figure 1, about 20% of the centers seem to be damaged by the Ca^{2+} depletion treatment, behaving like Tris-washed PS-II. In such centers, donation from TyrZ to P_{680}^+ is expected to take place in about 10–20 μs at pH 6.5 (Conjeaud & Mathis, 1980). The kinetics of P_{680}^+ reduction might be expected to show such a phase which is insensitive to flash number and Ca^{2+} recon-

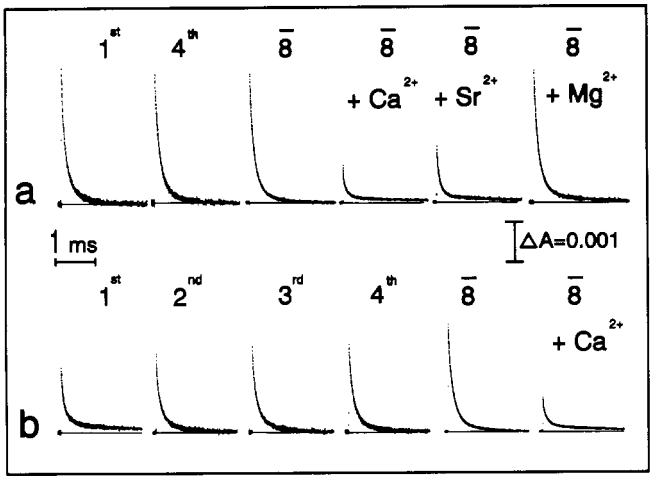

FIGURE 2: Amplitudes and reduction kinetics of P_{680}^+ in Ca^{2+}-depleted samples. Row a was recorded using dark-adapted NaCl–EGTA-treated/polypeptide-reconstituted PS-II, and row b was recorded using NaCl-washed PS-II. The traces show the absorption changes at 820 nm in the microsecond time range, associated with the reduction kinetics of P_{680}^+ following each flash of a sequence given to dark-adapted samples. The number of the flash is given above the corresponding trace. The symbol $\bar{8}$ indicates that the signal results from the averaging of eight successive flashes. In some cases, a second train of eight flashes was again averaged after the addition of 10 mM Ca^{2+}, Sr^{2+}, or Mg^{2+} (as chloride salts). The flash frequency was 1 Hz. Each row is the average of four experiments. PS-II concentration was 100 μg of Chl/mL. PS-II membranes were suspended in Chelex-treated medium containing 0.3 M sucrose, 10 mM NaCl, and 25 mM Mes, pH 6.5, for row a and 30 mM NaCl, 25 mM Mes, pH 6.5, and 50 μM EGTA for row b.

stitution. Indeed, in all the traces shown in Figure 2, with the exception of those taken on the first flash, the two faster phases account for 20–30% of the centers and are not affected by Ca^{2+} reconstitution. On the first flash, the 20–40-μs phase is much bigger than on subsequent flashes. The loss of the fast phases with further flashes is accompanied by a complementary increase in the slower phases. The slower phases probably correspond to the charge recombination between P_{680}^+ and Q_A^-. The 150–200-μs and 1-ms phases may correspond to recombination between P_{680}^+ and Q_A^- with Q_B reduced or Q_B oxidized, respectively [Ford & Evans, 1985; see, however, Gerken et al. (1989)]. Since, in this sample, the first four flashes converted the S_2 into the S_3 state (Boussac et al., 1990b), it is reasonable to suggest that the extra 20–40-μs phase, which is present after one flash but lost after the following flashes, is due to electron donation from the donor side of PS-II to P_{680}^+. Therefore, in this sample, the charge recombination between P_{680}^+ and Q_A^- may be competitive with electron donation to P_{680}^+. This may explain why four flashes are required to reach the S_3 state in all of the centers in this preparation (Boussac et al., 1990a).

The same experiment, done on NaCl-washed membranes without polypeptide reconstitution, is shown in row b of Figure 2. Unlike the situation with NaCl–EGTA-treated/polypeptide-reconstituted sample, a submicrosecond phase can be observed in the absence of Ca^{2+} and Sr^{2+}. The amplitude of P_{680}^+ detected on the first flash corresponded to ≈0.5 P_{680}^+ per PS-II. When 8 flashes were averaged in the absence of Ca^{2+}, about 0.8 P_{680}^+ per PS-II was detected. After Ca^{2+} addition, P_{680}^+ was reduced in the submicrosecond time range in about 75% of the PS-II centers. Amplitudes of the signals and reduction kinetics are presented in Table I. The main flash number related changes concerned the 150–200-μs phase which significantly increased after the first flash at the expense of the submicrosecond phase. Again this is attributed to charge recombination occurring when reduction of P_{680}^+ by the donor side becomes inhibited. The residual fast phase, which is unaffected by flash number or Ca^{2+} addition, is attributed to P_{680}^+ reduction by TyrZ in damaged centers. In the NaCl-washed PS-II membranes, approximately 20–25% of the centers are still functional after salt washing (Boussac et al., 1985a). The presence of these centers explains the observation that about 20–25% of P_{680}^+ reduction kinetics occurred in a submicrosecond range (row b of Figure 2) even after several flashes.

Slower TyrZ donation to P_{680}^+ may be difficult to distinguish from the $P_{680}^+Q_A^-$ recombination, and a combination of the two kinetics may be indistinguishable [see the situation in the presence of acetate reported by Saygin et al. (1986)]. Such problems make the kinetic assignments given in this section less than definitive. However, since the interpretations are based on the known charge storage characteristics in these preparations (Boussac et al., 1989, 1990a,b), they are probably justified.

Cl^- Depletion

EPR at Low Temperature. Figure 3 shows EPR spectra recorded from a sample which was Cl^--depleted in the presence of sulfate at pH 7.5 using the protocol as in Ono et al. (1986a). Spectrum a corresponds to the light-minus-dark spectra induced by 198 K illumination. As already reported (Ono et al., 1986a), no multiline signal corresponding to the S_2 state was observed. The addition of Cl^- in the dark to this sample, when thawed after the 198 K illumination, induced the formation of the multiline signal (not shown). A similar result was reported earlier in experiments in which Cl^- was added flash excitation (Ono et al., 1986a). Spectrum b corresponds to the signal induced by illumination of a Cl^--reconstituted sample at 198 K. The signal is the characteristic multiline signal arising from the Mn cluster in the S_2 state. Spectrum c in Figure 3 was recorded from a Cl^--depleted sample which was illuminated at 0 °C and then rapidly frozen. A narrow light-induced signal, with a peak to trough of less than 90 G, appeared at $g = 2$. Due to the overlap of the large signal arising from $TyrD^+$, it is not possible to obtain better resolution of this signal. The inset in Figure 3 shows the light-minus-dark signal induced by two flashes at room temperature given to the dark-adapted Cl^--depleted sample (spectrum e). Spectrum d shows the spectrum arising from the S_1 state of the Cl^--depleted sample. Examination of unpublished data taken in the course of an earlier EPR study (Ono et al., 1986a) done under the same conditions (i.e., two or more flashes) showed comparable results except that the signal was less well-resolved due to the use of a higher modulation amplitude (Ono, Zimmermann, and Rutherford, unpublished results). Spectrum f in Figure 3 shows a comparison made with the flash-induced signal in the Ca^{2+}-depleted sample taken from Boussac et al. (1990a). We conclude that Cl^--depletion of PS-II at pH 7.5 in the presence of sulfate also results in the formation of a light-induced EPR signal. This signal is attributable to a formal S_3 state on the basis of its illumination dependence similarities with the S_3 signal in the Ca^{2+}-depleted sample. This result and conclusion are similar to those of Baumgarten et al. (1990), who used F^--treated PS-II. In this case, however, it was claimed that the S_3 EPR signal had the same width as that seen in Ca^{2+}-depleted material.

It seems then that Cl^- depletion results in inhibition occurring after the formation of a formal S_3 state. In this way, this type of inhibition resembles that seen with Ca^{2+}-depleted PS-II. We therefore extended the comparison by investigating $TyrZ^+$ and P_{680}^+ kinetics in Cl^--depleted samples, in experi-

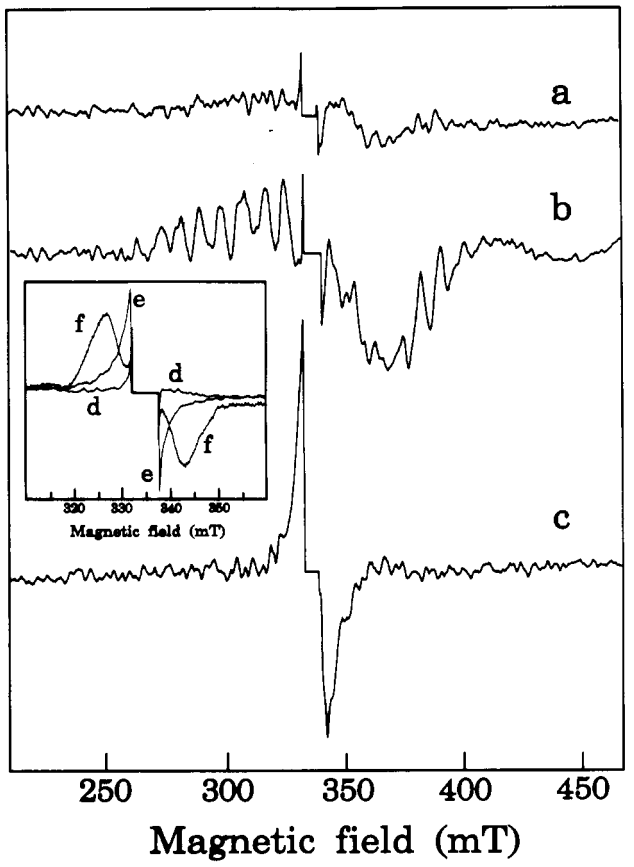

FIGURE 3: Low-temperature EPR spectra of Cl^--depleted samples. Spectra a and b are the light-minus-dark spectra after 198 K illumination in the absence of Cl^- (spectrum a) or after reconstitution with 100 mM NaCl before freezing of the sample (spectrum b). Spectrum c is a light-minus-dark spectrum after 1 min of illumination at 0 °C followed by rapid freezing at 198 K of the Cl^--depleted sample. Spectrum d, in the inset, corresponds to the dark spectrum in the Cl^--depleted sample. Spectrum e corresponds to the light-minus-dark spectrum after illumination by two flashes at room temperature of a Cl^--depleted sample. Spectrum f is the S_3 signal in a Ca^{2+}-depleted sample [see Boussac et al. (1990a) for the conditions]. Due to the overlap with the large signal arising from $TyrD^+$, the central part of the spectrum was deleted. Instrument settings: temperature, 10 K; microwave power, 20 mW for spectra a–c and 10 mW for spectra d and e; modulation amplitude, 22 G for spectra a–c and 10 G for spectra d and e; Chl concentration, 3 mg/mL. The Cl^--depleted samples were used in medium containing 0.3 M sucrose, 50 mM Na_2SO_4, and 50 mM HEPES pH 7.5.

ments analogous to those done on Ca^{2+}-depleted samples in Figures 1 and 2.

Kinetic EPR Measurements of $TyrZ^+$. Figure 4 shows the EPR signal arising from $TyrZ^+$ and its subsequent reduction in the dark in Cl^--depleted samples (traces b). Comparison can be made with Tris-washed samples diluted in the corresponding media (traces a). Our results agree with previous reports (Babcock et al., 1984) in which only 30% of $TyrZ^+$ could be detected by flash illumination in the absence of Cl^- [see also Franzen et al. (1985)]. Addition of Cl^- led to a decrease in the amplitude of the $TyrZ^+$ signal which is detectable in the millisecond time range (traces c in Figure 4). The low yield of $TyrZ^+$ formed in the absence of Cl^- led us again to record P_{680}^+ reduction in Cl^--depleted samples. The significance of the two panels A and B in Figure 4 will be explained below.

Flash-Induced Absorption Studies of P_{680}^+. Figure 5 shows absorption changes in the microsecond time range arising from P_{680}^+ formation and reduction in Cl^--depleted samples. The P_{680}^+ reduction kinetics were recorded after the first, second,

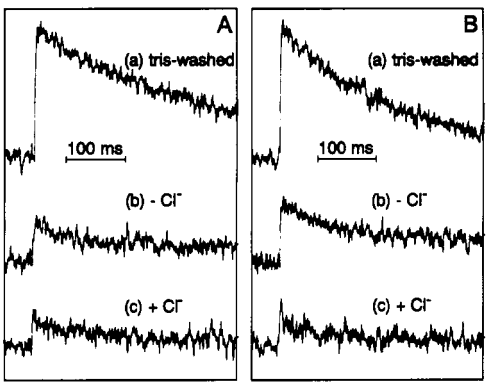

FIGURE 4: Reduction kinetics of $TyrZ^+$ in Cl^--depleted samples. Amplitudes of the EPR signal arising from $TyrZ^+$ induced by flash illumination and kinetics of the subsequent dark-reduction in Tris-washed PS-II samples (traces a) or in Cl^--depleted samples (traces b). Traces c correspond to Cl^--depleted samples resuplemented with 100 mM NaCl. PS-II membranes were suspended in a Chelex-treated medium containing 0.3 M sucrose, 50 mM Na_2SO_4, and 50 mM HEPES, pH 7.5 (panel A). For the experiments in panel B, the medium contained 0.3 M sucrose, 50 mM Na_2SO_4, 10 mM $Ca(OH)_2$, and 50 mM HEPES, pH 7.5. Each trace is the average of 512 scans (4 samples and 128 scans per sample). The flash spacing was 4 s, and the signal was recorded for 400 ms. Instrument settings: temperature, ≈20 °C; microwave frequency, 9.85 GHz; modulation amplitude, 6.3 G; time constant, 1.25 ms; microwave power, 20 mW; chlorophyll concentration, 1.5 mg/mL. The traces were recorded at a magnetic field close to the low-field maximum of $TyrZ^+$.

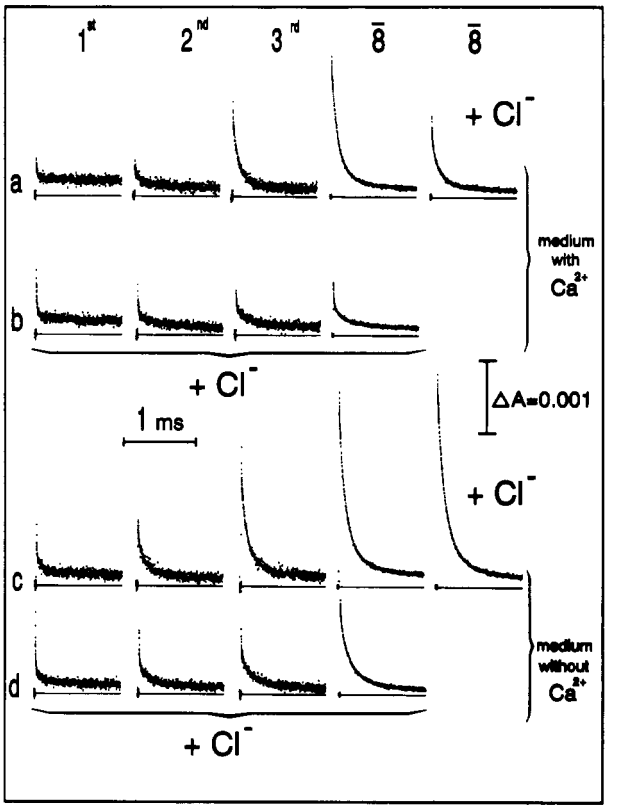

FIGURE 5: Amplitudes and reduction kinetics of P_{680}^+ in Cl^--depleted samples. The traces show the absorption changes at 820 nm, in the microsecond time range, associated with the reduction kinetics of P_{680}^+ following each flash of a sequence given on dark-adapted Cl^--depleted PS-II (rows a and c). When indicated, 100 mM NaCl was added. For rows b and d, 100 mM NaCl was added in the medium before the addition of the membranes. The media used with or without Ca^{2+} were the same as those used in Figure 4. All other experimental conditions were similar to those in Figure 2.

and third flashes (1 s apart) given to a dark-adapted sample diluted in Cl^--free medium (rows a and c) or in Cl^--containing

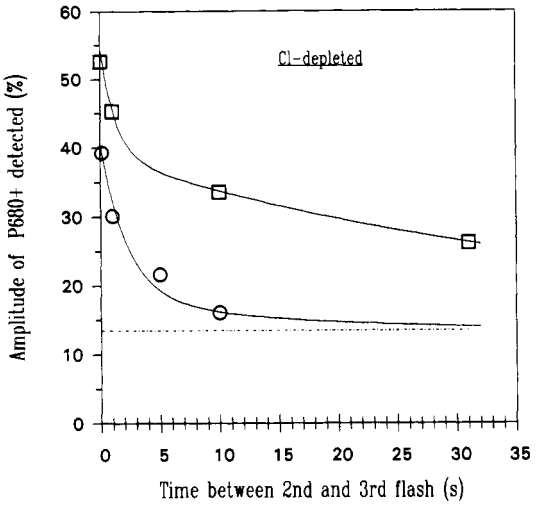

FIGURE 6: Lifetime of the species oxidized by two flashes in Cl^--depleted samples. The experiment described in Figure 5 was repeated by varying the dark time between the second and third flashes. Then the total amount of P_{680}^+ decaying in the microsecond time range after the third flash was obtained from the fitting procedure. This value is plotted versus the dark time between the second and third flashes. All the experimental conditions are identical to those in Figure 5. The experiment was done in the presence (○) or in the absence (□) of 10 mM $Ca(OH)_2$ in the medium. The horizontal dotted line corresponds to the amount of P_{680}^+ detected after the second flash in the presence of Ca^{2+} (in the absence of Ca^{2+}, the amplitude is slightly lower by ≈15%) and may be considered as the asymptotic level of the kinetics. The shortest dark times used were 25 ms in the presence of Ca^{2+} and 150 ms in the absence of Ca^{2+}. For these two points, a mixture of 0.5 mM PPBQ and 1 mM ferricyanide was used as electron acceptors.

medium (rows b and d). For rows b and d, 100 mM NaCl was added to the dilution medium before the addition of the membranes. In each experiment, after the third flash, the signal was averaged over the eight following flashes. For rows a and c, 100 mM NaCl was then added, and the signal was again averaged over eight flashes. As already observed (Ono et al., 1986b), only a small signal was observed on the first two flashes in all the conditions. This indicates that P_{680}^+ reduction kinetics mainly occurred in the submicrosecond time range in the S_1 and S_2 states in centers inhibited due to Cl^- depletion. This result contrasts with those obtained for Ca^{2+}-depleted PS-II (see Figure 2). In the absence of Cl^- (rows a and c), the extent of P_{680}^+ decaying in the microsecond time range strongly increased on the third flash, reaching about 70% of the signal averaged over the eight following flashes in the absence of Cl^-. Results of the kinetics fitting procedure in Cl^--depleted samples are shown in Table I. In contrast with the Ca^{2+}-depleted samples, the best fit was obtained with two phases. As shown in Table I, the variation in the P_{680}^+ reduction kinetics concerned mainly the 60–70-μs phase which gradually appeared during the first flashes at the expense of the submicrosecond phase. The millisecond phase was only weakly dependent on flash number.

The increase in the amount of P_{680}^+ detected in the microsecond time range after the third flash in the absence of Cl^- (rows a and c in Figure 5) reflects a major change in the electron donation kinetics of P_{680}^+ in comparison with the reduction kinetics of P_{680}^+ after the second flash. To determine the lifetime of the oxidized state, formed on the second flash, which is responsible for the modification of P_{680}^+ reduction kinetics on the third flash, we performed an experiment in which the time between the second and third flash was varied. The result of such an experiment is plotted in Figure 6. Recovery of a level similar to that obtained after the second flash is apparently polyphasic, and in about half of the centers, the recovery is slower than 1 s. This suggests that, in about half of the centers, the stabilized state after the second flash is not S_2TyrZ^+ since $TyrZ^+$ is unlikely to be so long-lived and we know that S_3 is formed in a proportion of centers in these conditions (Figure 3). Moreover, no such kinetics for $TyrZ^+$ reduction were observed in the EPR experiments (Figure 4). However, this can only partially account for the fraction of the centers recovering in the millisecond time scale in the experiment in Figure 6. It seems possible that part of the rapid phase of recovery may originate in centers in which TyrD was in the reduced form in the dark. Indeed, a lower amplitude of $TyrD^+$ was present in the dark-adapted Cl^--depleted samples [not shown, but see Damoder et al. (1986)]. Electron donation from TyrD to $TyrZ^+$ (or S_3?) after the second flash could result in the rapid reappearance of TyrZ, capable of P_{680}^+ reduction in the submicrosecond time range. TyrD donation to S_2 and S_3 has been well characterized in functional PS-II [see Vass and Styring (1991) and references cited therein], the kinetics ranging from 0.4 to 20 s depending on the pH (Vass & Styring, 1991). The fact that S_2 is more stable in Cl^--depleted material (Ono et al., 1986a) probably favors TyrD oxidation after the second rather than after the first flash. It is also relevant to note that TyrD oxidation can occur in the millisecond time range when the electron donor side is inhibited (Buser et al., 1990).

Effect of Ca^{2+} in the Buffer. The effect of Cl^- reconstitution was found to be strongly dependent on the presence or the absence of Ca^{2+} in the medium. When Ca^{2+} was present in the medium (Figure 5, rows a and b), addition of Cl^- reconstituted a submicrosecond reduction rate of P_{680}^+ (row a). When Cl^- was added to Cl^--depleted samples which had been preilluminated in the absence of Ca^{2+}, no restoration of the submicrosecond phase was observed (row c). Figure 6 shows that the lifetime of the species oxidized by the second flash in the absence of Cl^- is longer in the absence of Ca^{2+} than in its presence, with a slow phase of about 30 s.

The oxygen-evolving activity of Cl^--depleted samples was ≈50 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} in Ca^{2+}-free media and ≈0 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} in the presence of 10 mM Ca^{2+} in the medium. The activity of Cl^--reconstituted sample measured in Ca^{2+}-containing medium was ≈550 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} under saturating light intensity whereas the activity was ≈350 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} in Ca^{2+}-free medium. Measurements at different, nonsaturating light intensities in the presence or the absence of Ca^{2+} in the medium showed that, in the absence of added Ca^{2+}, all the centers functioned but at a slower rate (not shown). This slowdown in oxygen evolution may correlate with the absence of the submicrosecond reduction kinetics of P_{680}^+ after addition of Cl^- in Ca^{2+}-free medium (row c in Figure 5). This Ca^{2+} effect seems specific since addition of 10 mM $MgSO_4$ did not restore submicrosecond reduction kinetics of P_{680}^+ (not shown). Sr was not tested due to the very low solubility of $SrSO_4$.

From Figure 5, it appears that the presence of Ca^{2+} is required for the recovery of submicrosecond donation to P_{680}^+ upon Cl^- reconstitution after the sample is preilluminated by a few flashes. If Cl^- is added before the first flash, the effect of Ca^{2+} on P_{680}^+ reduction is much less marked (rows b and d in Figure 5). The behavior of $TyrZ^+$ in Cl^--depleted samples is only slightly modified by the presence or the absence of Ca^{2+} in the medium (Figure 4). It is known that PS-II centers are much more susceptible to damage by light when the electron donor side is blocked. This effect is known as weak-light photoinhibition (Callahan et al., 1986; Hind et al., 1969;

Jegerschöld et al., 1990; Theg et al., 1986). To rule out an effect of weak-light photoinhibition, we also checked the effect of preillumination upon a Cl^--depleted sample. Cl^--depleted samples were first preilluminated at a saturating light intensity for 5 s in the presence or the absence of Ca^{2+} in the medium. Then 100 mM NaCl was added, and the oxygen evolution activity was measured. The extent of the inhibition induced by the preillumination, when compared to a nonpreilluminated sample before the addition of Cl^-, was less than 20% and was similar in the presence or the absence of Ca^{2+}. In our conditions, the effect of preillumination on the Cl^--reconstitutable P_{680}^+ reduction kinetics, in the absence or the presence of Ca^{2+}, is clearly unrelated to this photoinhibition.

The relationship between this kind of Ca^{2+} effect to that generally studied is not clear, but it seems likely that the Ca^{2+} site involved is different from that responsible for the inhibition of S-state turnover after S_3 as characterized earlier. The reasons for this assertion are the following: (1) When this new Ca^{2+} effect is manifest, Cl^- addition reconstitutes activity up to 65% of the untreated sample while the well-known Ca^{2+} effect induced by salt or citrate washing is not reactivated by Cl^- (Cammarata & Cheniae, 1987; Ono & Inoue, 1988). (2) Unlike the Ca^{2+} effect characterized in NaCl-washed Ca^{2+}-depleted PS-II, the Ca^{2+} effect noted here does not block the S-state cycle. The inclusion of these results in the paper is for the sake of completeness, for the benefit of those wishing to reproduce the data and because the states formed seem quite unusual. Further work is required before the significance of these phenomena can be assessed.

Discussion

Calcium Depletion. From earlier work, it is known that charge accumulation in Ca^{2+}-depleted PS-II can advance as far as the S_3 state before inhibition occurs (Boussac et al., 1985b, 1989; Boussac & Rutherford, 1988a). In the repetitive flash EPR experiment in Figure 1, potential information on TyrZ chemistry occurring on the first few flashes is lost due to the averaging of a large number of flashes. This experiment, however, provides data on the reduction and oxidation of TyrZ after formation of the S_3 state in Ca^{2+}-depleted PS-II. Contrary to our expectations, the experiment showed that no $TyrZ^+$ was formed under these conditions.

The flash absorption studies of P_{680}^+ reduction (Figure 2 and Table I) show that in Ca^{2+}-depleted PS-II, reduction of P_{680}^+ occurs in the microsecond time range and is much slower than in Ca^{2+} (and Sr^{2+})-reactivated PS-II. After formation of S_3, P_{680}^+ was mainly reduced with kinetics in the 150–200-μs and 1-ms time ranges. These kinetics correspond to those expected for P_{680}^+ reduction by charge recombination with Q_A^-. This is consistent with the EPR data of Figure 1 indicating that $TyrZ^+$ is not formed under these conditions.

The lack of TyrZ oxidation in the Ca^{2+}-depleted PS-II apparently contradicts the conclusions drawn earlier from luminescence experiments in which it was reported that Ca^{2+} depletion resulted in inhibition occurring after formation of S_3TyrZ^+ (Boussac et al., 1985b). In light of the more direct evidence presented here, we have reassessed the earlier data. The luminescence amplitude at 2 ms after the flash was found to vary with flash number, showing a maximum corresponding to the S_3TyrZ^+ state in Ca^{2+}-reconstituted samples. In the Ca^{2+}-depleted material, a slightly enhanced maximum was reached on the third flash, and little variation occurred on subsequent flashes. This was interpreted as being due to inhibition of electron transfer after S_3TyrZ^+ was formed (Boussac et al., 1985b). However, since the luminescence emission in oxygen-evolving PS-II represents only a very small fraction of the centers (accounting for part of the so-called miss factor), an electron-transfer block at this level should have given rise to a much greater enhancement of the luminescence amplitude. It seems likely from the present work that $P_{680}^+Q_A^-$ recombination takes place after S_3 is formed; thus, an increase in luminescence in the 200-μs time range would be expected. Indeed, a reexamination of the original data shows that, although the measurements made at 200 μs were found to be less directly related to the enzyme cycle and more severely influenced by instruments artifacts, Ca^{2+} depletion results in a much greater stimulation of the third flash luminescence at 200 μs than that at 2 ms (A. Boussac, unpublished results). Thus, we can conclude that the luminescence data can be reconciled with the current data and interpretation.

The inhibition of electron transfer between TyrZ and P_{680}^+ in Ca^{2+}-depleted PS-II was already reported in cyanobacteria (Satoh & Katoh, 1985). It seems reasonable to propose that Ca^{2+} depletion in cyanobacteria leads to the same lesion as in plants, i.e., inhibition of the $S_3TyrZP_{680}^+$ to $S_3TyrZ^+P_{680}$ transition. This is significant since Ca^{2+} depletion in cyanobacteria is usually considered to be different from that in plants [e.g., see Yocum (1991) and Debus (1991)].

In earlier work, the UV–visible spectrum of the S_2 to S_3 transition in Ca^{2+}-depleted PS-II showed an electrochromic shift which was taken as an indication that the oxidation was not accompanied by a compensating deprotonation (Boussac et al., 1990a). This electrochromic shift in the S_2 to S_3 transition is normally absent in control PS-II in which proton release occurs in this transition (Dekker et al., 1984c; Lavergne, 1987). This observation provides a possible explanation for the electron-transfer lesion. The presence of an extra positive charge in the S_3 state in Ca^{2+}-depleted PS-II could have an electrostatic effect on TyrZ, preventing its oxidation by P_{680}^+. A role for Ca^{2+} in the deprotonation reactions associated with water oxidation was suggested earlier (Boussac & Rutherford, 1988c).

In Ca^{2+}-depleted PS-II, the reduction of P_{680}^+ was found to be slow even upon the first few flashes given to dark-adapted material, i.e., in reactions where electron donation and charge storage on the donor side are known to occur. This effect of Ca^{2+} depletion may reflect another electrostatic limitation associated with the inhibition of deprotonation at earlier states in the enzyme cycle, but other explanations cannot be excluded.

A greater fraction of centers in which P_{680}^+ decays in the submicrosecond range is seen in NaCl-washed membranes on the first few flashes, and this phase is converted to a 150–200-μs phase after a few flashes. This contrasts with the virtual absence of submicrosecond P_{680}^+ reduction kinetics in Ca^{2+}-depleted material which had been treated with EGTA and reconstituted with the 17- and 23-kDa polypeptides. In salt-washed samples, treated in the light with a high concentration of EGTA but lacking the 23- and 17-kDa polypeptides, the P_{680}^+ reduction kinetics were similar (not shown) to those in the salt-washed material (Figure 2, row B). Thus, the absence of submicrosecond kinetics on the first few flashes in dark-adapted Ca^{2+}-depleted samples seems to be associated with the reconstitution of the 17- and 23-kDa polypeptides. Evidence that the binding of the extrinsic polypeptides influences the environments of the redox components has already been presented (i.e., modification of the width of the S_3 EPR signal) (Boussac et al., 1990b).

The kinetic EPR experiments of Figure 1 showed that $TyrZ^+$ reduction was abnormally slow in the sample reconstituted with Sr^{2+}. This observation adds to the list of modifications induced by Sr^{2+} reconstitution in place of Ca^{2+}.

First, oxygen-evolving activity is decreased, but although all the centers function, they do so less efficiently (Boussac & Rutherford, 1988b). This may be related to the previous observation that the S_2 to S_3 transition was less efficient in the Sr^{2+}-reconstituted sample (Ono & Inoue, 1989) and to the present work in which we report a slowdown in $TyrZ^+$ reduction on at least two S-state transitions resulting in less efficient O_2 evolution in such a preparation. Second, the Mn multiline signal is modified, presumably due to a structural change affecting the environment of the Mn (Boussac & Rutherford, 1988a). It seems likely that the reactions which are affected are the S_3TyrZ^+ to S_0TyrZ and the S_2TyrZ^+ to S_3TyrZ transitions, but this remains to be verified experimentally. As well as a structural change (or perhaps as a result of such a change), the Sr^{2+}-reconstituted PS-II may suffer from a lesion (a slowdown?) at the level of the deprotonation reactions, i.e., a less severe case of the disorder afflicting the Ca^{2+}-depleted material.

Chloride Depletion. Prompted by the observation of an EPR signal in Cl^--depleted/F^--treated PS-II (Baumgarten et al., 1990) which seemed to be similar to the S_3 signal that we had characterized previously in Ca^{2+}-depleted material, we tested for the presence of such a signal in Cl^--depleted/SO_4^{2-}-treated PS-II. This type of Cl^- depletion has been more generally used in the literature. An EPR signal attributable to S_3 was indeed observed after continuous illumination and after two or more flashes (Figure 3). The splitting of the signal is significantly narrower than that seen in Ca^{2+}-depleted material, presumably reflecting a weaker magnetic interaction between the histidine radical and the Mn cluster according to the model of Boussac et al. (1990a).

We suggested earlier that the S_3 state in the functional enzyme could also consist of a His radical interacting with the manganese cluster but that the resulting signal was undetectable (Boussac et al., 1989, 1990a,b). The greatly narrowed S_3 signal seen in the Cl^--depleted sample encourages us in this view since a further narrowing of the signal in the intact system would lead to the signal becoming undetectable.

The repetitive flash EPR experiment done on Cl^--depleted PS-II (Figure 4) shows that $TyrZ^+$ decaying in the millisecond time range is absent in more than 60% of the centers. In the same samples, after a few flashes, P_{680}^+ seems to be reduced by charge recombination with an electron on the acceptor side (Figure 5). Since we know (Figure 3) that after two flashes the Cl^--depleted samples exhibit an EPR signal attributable to the S_3 state in the Ca^{2+}-depleted sample, we may propose that in the majority of the centers TyrZ cannot be oxidized after S_3 formation.

A block after S_3 formation in Cl^--depleted PS-II agrees with the proposition of Homann et al. (1986) but disagrees with Theg et al. (1984), Itoh et al. (1984), and Ono et al. (1986a,b) (see the introduction). In the following, we attempt to rationalize these conflicting reports.

Theg et al. (1984) recorded luminescence emission in the hundreds of microseconds time range, corresponding to charge recombination between P_{680}^+ and Q_A^-, after each flash of a sequence given to a dark-adapted Cl^--depleted PS-II. An increase of the luminescence was observed after the third flash, indicating that only two charges could be accumulated in Cl^--depleted material. By measuring the rise of chlorophyll fluorescence in the microsecond time range attributed to the reduction of P_{680}^+, Itoh et al. (1984) also concluded that Cl^- depletion resulted in inhibition after two charges were accumulated on the donor side. In a study using flash absorption spectroscopy, by varying the time between the second and third flashes given to a dark-adapted Cl^--depleted sample, Ono et al. (1986b) observed that the rate of recovery of P_{680}^+ reduction in the submicrosecond time range seemed to match the $TyrZ^+$ reduction kinetics found in Tris-washed PS-II. However, a slower phase occurring in the seconds time scale was reported as a preliminary result. In Figure 6, we show that an important slow recovery phase exists. In addition, a corresponding slow phase in the recovery of submicrosecond P_{680}^+ reduction was observed in a large fraction of PS-II centers by Itoh et al. (1984) using fluorescence.

The data in these three earlier studies are in fact at least as easily reconciled with inhibition occurring between TyrZ and P_{680}^+ after S_3 formation as with inhibition at the S_2TyrZ^+ to S_3TyrZ transition. All three groups, however, concluded that the latter was inhibited.

The EPR experiments, in which the Mn multiline signal was used to monitor S-state turnover, were also interpreted as indicating that inhibition occurred after S_2 formation (Ono et al., 1986a). This reinforced the earlier conclusions that S_3 was not formed and was influential in the interpretation of the subsequent optical (Ono et al., 1986b) and thermoluminescence studies (Vass et al., 1987; Inoue, 1987). However, the Mn signal monitored had to be generated by postflash addition of Cl^-. From the present work, it seems likely that the extra time needed for mixing the sample with the Cl^- solution allowed the modified S_3 state to deactivate back to the S_2 state, resulting in no indication of S-state turnover after S_2 formation (this suggestion was originally made to us by R. Debus, University of California, Riverside).

It seems clear then that all of the earlier conflicting reports can be easily reconciled with the conclusion from the current work that Cl^- depletion in the presence of sulfate results in the inhibition of electron transfer from TyrZ to P_{680}^+ after S_3 has been formed. The conclusion that S_3 can be formed in such material agrees with the original deductions of Homann et al. (1986) based on a thermoluminescence study. Interestingly, Homann et al. (1986) showed that rapid addition of Cl^- to the sample after formation of the modified S_3 state resulted in the formation of a normal S_3 state capable of normal enzyme cycle reactions, presumably resulting in water oxidation. Similar experiments in Ca^{2+}-depleted PS-II (Boussac & Rutherford, 1988b) were taken as support for the proposal that S_3 in the inhibited enzyme was the same chemical species as S_3 in the functional enzyme (Boussac et al., 1990b).

Detailed studies of cyanobacterial PS-II inhibited by the presence of a high concentration of acetate have been reported (Saygin et al., 1986; Bock et al., 1988; Gerken et al., 1989). The phenomenology reported is strikingly similar to the situation reported in the present work. In the acetate-treated PS-II, two interesting observations remained unexplained: (1) the identity of an unknown electron donor to P_{680}^+ (Gerken et al., 1989) and (2) the origin of the unusual broadening of the EPR signal arising from P_{680}^+ when recorded under conditions in which the electron donation is blocked (Brok et al., 1988). In light of the present results, it seems reasonable to suggest that His is the unknown donor and that the His radical is the species responsible for broadening the P_{680}^+ EPR signal. If the latter suggestion is correct, a dipolar broadening of the magnitude reported indicates a $His^\bullet$ to P_{680}^+ distance of 8.5 Å [calculated according to Hogansson and Babcock (1989)].

It is well-known that electron-transfer inhibition on the electron donor side leads to enhanced photolability of PS-II (Callahan et al., 1986; Theg et al., 1986). Since Cl^- depletion is a convenient reversible method of inhibiting the donor reactions of PS-II, it has been used in some studies of weak-light

photoinhibition (Jegerschöld et al., 1990; Hind et al., 1969; Theg et al., 1986). It was suggested that $TyrZ^+$ could be the initial target of photodamage. From the present results, it seems that $TyrZ^+$ is not formed in the majority of centers under these conditions. What is formed is a long-lived histidine radical (Boussac et al., 1990a) and P_{680}^+. Either or both of these could be an active species in photoinhibition. The Tyr radical could of course be the active species in those centers in which it is formed.

It seems that Cl^- depletion and Ca^{2+} depletion result in an essentially similar electron-transfer block. We suggested above that the Ca^{2+} depletion lesion may be due to the inhibition of Tyr oxidation due an electrostatic effect associated with the presence of a net positive charge from S_3. It is possible then that the lesion in Cl^--depleted PS-II has a similar origin. This implies that a role of Cl^- is in the charge compensation reactions associated with the deprotonation events. This kind of role for Cl^- has been discussed earlier (Coleman, 1990; Homann, 1987; Rutherford, 1989). The interrelationships between the roles of Cl^- and Ca^{2+} have been noted earlier (Coleman, 1990; Homann, 1987), and a speculative model involving Cl and H_2O exchange between Ca and the Mn cluster has been discussed. It was pointed out earlier that this model does not exclude a role for Ca^{2+} (and Cl^-) in deprotonation reactions (Rutherford et al., 1992).

In the O_2-evolving enzyme, the oxidation of two molecules of water to molecular oxygen does not seem to occur before all four positive charge equivalents have been accumulated [see Rutherford et al. (1992) for a review]. The concentration of four positive charges in the immediate proximity of the active site would certainly cause serious electrostatic restraints on the redox reactions; e.g., the electron-transfer reactions would be retarded and eventually become thermodynamically unfavorable. It seems likely that such problems are minimized in the functional enzyme by compensatory deprotonation reactions, possibly by the binding of counterions, and perhaps by the distribution of the oxidizing equivalents among different carriers (i.e., the Mn atoms of the cluster and the aromatic amino acids). From the present work, we propose that these processes may involve mechanisms in which both Ca^{2+} and Cl^- play important roles.

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Sequential Resonance Assignments of Oxidized High-Potential Iron–Sulfur Protein from *Chromatium vinosum*†

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ABSTRACT: 2D NMR spectra of the high-potential iron–sulfur protein (HiPIP) from *Chromatium vinosum* have been used to obtain partial resonance assignments for the oxidized paramagnetic redox state of the protein. Sequence-specific assignments were made using NOESY and COSY spectra in H_2O and D_2O of the following backbone segments: Asn-5–Arg-33, Glu-39–Asp-45, Gly-55–Cys-63, Gly-68–Ala-78, and Leu-82–Gly-85. NOESY spectra with a spectral width wide enough to include the hyperfine-shifted resonances revealed numerous NOE contacts between these signals and those in the main envelope of the proton spectrum. With the aid of the X-ray crystal structure [Carter, C. W., Kraut, J., Freer, S. T., Xuong, N. H., Alden, R. A., & Bartsch, R. G. (1974) *J. Biol. Chem. 249*, 4212], these NOEs permitted seven of the nine hyperfine-shifted signals to be assigned to three of the cysteine residues liganded to the metal cluster (Cys-43, Cys-46, and Cys-77). The other two hyperfine-shifted signals produced no detectable NOEs to other resonances in the spectrum and were tentatively assigned to the remaining cysteinyl ligand (Cys-63). These assignments, in conjunction with recent theoretical models of the electronic structure of the Fe_4S_4 cluster [Noodleman, L. (1988) *Inorg. Chem. 27*, 3677; Bertini, I., Briganti, F., Luchinat, C., Scozzafava, A., & Sola, M. (1991) *J. Am. Chem. Soc. 113*, 1237], indicate that the iron atoms coordinated to Cys-63 and Cys-77 are those of the mixed-valence Fe^{3+}–Fe^{2+} pair whereas Cys-43 and Cys-46 are ligands to the Fe^{3+}–Fe^{3+} metal pair.

Iron–sulfur proteins play important roles in terminal respiration, photosynthesis, and nitrogen fixation and are of particular structural interest because of the wide range of redox potentials they can adopt. Those containing one or more Fe_4S_4 clusters have the most complex electronic structures and are grouped into two classes according to their one-electron redox potentials and the $[Fe_4S_4]^{n+}$ core oxidation levels between which they interconvert ($n = +1/+2$ or $+2/+3$) (Palmer et al., 1975; Carter, 1977a). High-potential iron–sulfur proteins (HiPIPs) have relatively large positive reduction potentials of up to 350 mV and interconvert between the $+2/+3$ states while the low potential ferredoxins ($E°$ ca. −400 mV) shuttle between the $+1/+2$ states. Despite the existence of X-ray crystal structures for HiPIP from *Chromatium vinosum* (Carter et

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