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Ca^{2+} depletion modifies the electron transfer on both donor and acceptor sides in Photosystem II from spinach

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Abstract

Ca^{2+} depletion of Photosystem II from spinach results in reversible retardation of electron transfer on both donor and acceptor sides. On the donor side, a decrease of the electron transfer rate from TyrZ results in an enhanced charge recombination between the oxidized primary donor, $P680^+$, and the reduced acceptor quinone, Q_A^-, which in turn leads to a decrease in the amplitude of the fluorescence yield. In addition, slow electron transfer from the manganese cluster in the dark-stable S_2 state results in the appearance of a transient EPR signal from $TyrZ^{ox}$ which decays with half-times of 600 ms and 5 s. On the acceptor side, the disappearance of the 400 μs decay transient in the fluorescence yield indicates that the electron transfer from Q_A^- to Q_B has been severely inhibited. These results suggests that removal of a Ca^{2+} ion from the donor side in PS II, which results in the inhibition of oxygen evolution and in the appearance of an EPR signal in the S'_3 state leads to structural changes which are transmitted to the acceptor side. The strikingly similar behavior after depletion of Ca^{2+} of the $TyrZ^{ox}$ EPR signal and the split radical signal from the S'_3 state suggests that both signals involves the same oxidized amino acid residue, $TyrZ^{ox}$. The absence of large effects on the EPR properties of the non-heme iron suggests that the structural changes on the acceptor side are subtle in nature. Chemical modification of histidine results in inhibition of Q_A^- to Q_B electron transfer and to changes in the magnetic properties of the oxidized non-heme iron but only to minor perturbations of the donor-side. This suggests that histidine, susceptible to chemical modification, is located mainly on the acceptor side of PS II.

Keywords: Photosystem II; Calcium; Quinone; Electron transport; Tyrosine; Oxygen evolution

1. Introduction

In Photosystem II the light-induced oxidation of water is catalyzed by a charge-accumulating complex consisting of four manganese ions. During turnover, electrons are delivered from the complex via a redox-active tyrosine, TyrZ, to the oxidized reaction center chlorophyll, $P680^+$, to replace those transferred to the Photosystem II acceptor side. The repeated abstraction of electrons causes the manganese complex to go through a series of intermediates, denoted S_0 to S_4, in a cyclic fashion with evolution of molecular oxygen on the S_4 to S_0 transition.

There is considerable evidence that bound Ca^{2+} is necessary for functional photosynthetic oxygen evolution. (For recent reviews on the structure and function of PS II and the role of Ca^{2+}, see [1–4].) At least two Ca^{2+} ions are associated with PS II. A recent study of Ca^{2+} binding using a PS II core complex [5], has localized one Ca^{2+} to a binding site in the PS II light-harvesting complex, LHC II, while a second Ca^{2+} was associated with the PS II core. This Ca^{2+} appears to be identical to the bound Ca^{2+} in less resolved PS II preparations from which it can be extracted with chelators such as EGTA or citrate [6,7]. In NaCl-washed PS II membranes, a major part of the mobile Ca^{2+} is characterized by a dissociation constant of 50–100 μM and a minor fraction with the dissociation constant of about 1 mM [8]. Removal of Ca^{2+} from PS II has been reported to lead to impaired electron transfer from TyrZ to $P680^+$ [9] and to inhibition of S-transitions (reviewed in

Abbreviations: PS II, Photosystem II; MES, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*NNN'N'*-tetraacetic acid; PPBQ, phenyl-*p*-benzoquinone; DEPC, diethylpyrocarbonate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

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[1]). Particularly, Ca^{2+} depletion results in a dark-stable, modified S_2 state and an EPR detectable S_3 state with magnetic interaction between an amino acid radical and the manganese cluster [10–12]. In addition to the effects on the PS II donor side, there is also evidence of inhibition of acceptor side function after depletion of Ca^{2+}. In one early study, a binding site for Ca^{2+} between Q_A and Q_B was reported [13]. More recently, Krieger et al. [14] reported an increase in the midpoint potential of Q_A by 120 mV from fluorescence yield and thermoluminescence data after depletion of Ca^{2+}. Other thermoluminescence results, however, failed to confirm such a shift [15]. In this communication we have re-investigated the role of Ca^{2+} depletion on the donor and acceptor sides in PS II by its effects on electron transfer kinetics and spectroscopic properties of the acceptor side.

2. Materials and methods

Oxygen-evolving PS II membranes were prepared from hydroponically grown spinach [16] and suspended in 25 mM MES-NaOH (pH 6.5), 20 mM NaCl and 0.3 M sucrose.

Ca^{2+}-depleted PS II membranes lacking the 16 and 23 kDa polypeptides were prepared by washing with NaCl-EGTA as described in [17] and suspended in the above buffer containing 10 mM EGTA. After depletion of Ca^{2+} the O_2-evolution rate decreased to 15–20% of the original value (500–600 μmol O_2/(mg Chl) per h). Re-addition of Ca^{2+} (20 mM) led to a restoration to about 80% of the original activity.

Chemical modification of histidine with diethylpyrocarbonate (DEPC) was accomplished essentially as described by Tamura et al. [18] by treating the PS II membranes at 0.5 mg chlorophyll/ml with 3.5 mM DEPC in the suspension buffer at 20° C in the dark. After 1 h the modification reaction was stopped with 50 mM histidine and the PS II membrane was washed with twice with suspension buffer.

Room-temperature EPR measurements were measured at X-band with a Bruker ESP 300 instrument. Low-temperature EPR spectra were measured with a Bruker ER 200 D-SRC spectrometer equipped with an Oxford Instruments He-flow cryostat and temperature controller.

For EPR studies of flash-induced kinetics of tyrosine radicals a flat sample cell was used with PS II membranes at a concentration of about 1.5 mg chlorophyll/ml. Excitation flashes (8 ns, 300 mJ at 532 nm) were provided with a frequency-doubled YAG-Nd laser. The S'_3 state EPR signal was induced in the Ca^{2+}-depleted samples by flash illumination using the same laser or by 30 s continuous illumination with white light from a heat-filtered 800 W projector lamp. When indicated, samples used for EPR contained 0.5 mM phenyl-*p*-benzoquinone (added from a 20 mM solution in dimethylsulfoxide).

The decay kinetics of flash-induced fluorescence were measured with a PAM fluorimeter (Walz, Effeltrich, Germany) as described in [19]. The fluorescence yield kinetics were analyzed by least-square curve fitting, using three exponential components with free running parameters. Since the fluorescence yield is not linearly correlated to the redox states of Q_A, the relative Q_A^- concentration was estimated according to the model of Joliot and Joliot [20], using 0.5 for the value of the energy-transfer parameter between PS II units.

3. Results

3.1. Effect of Ca^{2+} depletion on fluorescence yield kinetics

Fluorescence yield kinetic studies of PS II, with the extrinsic 16 and 23 kDa polypeptides removed by washing with 1 M NaCl, show the presence of several decay transients with decay times of about 400–500 μs, 4 ms and a longer than 400 ms phase (Fig. 1, Table 1). Although the experiment was done using repetitive flashing, the interval of 20 s was long enough to allow the system to relax between each flash and to prevent accumulation of significant amounts of intermediates (see below). Therefore, in effect, each flash is equivalent to a 'first' flash given to dark-adapted material. The shortest phase (400 μs) represents forward electron transfer from Q_A^- to Q_B, while the 4 ms transient most likely is due to transfer from Q_A^- to the plastoquinone pool (rate-limited by the binding of plastoquinone to the Q_B site). The half-time of particularly the shortest transient is longer than what has been

Table 1
Effect of Ca^{2+} depletion on amplitudes and half-times of decay of flash-induced fluorescence transients in Photosystem II membranes

	Fluorescence transient					
	$t_{1/2}$ (μs)	amplitude [a]	$t_{1/2}$ (ms)	amplitude [a]	$t_{1/2}$ (ms)	amplitude [a]
Untreated	460	26	4.0	25	> 400	49
NaCl-washed	450	22	3.7	19	> 400	59
Ca^{2+}-depleted [b]	450	0	3.6	4.7	> 400	21.9
Ca^{2+}-reconstituted	400	19	4.5	18	> 400	52

[a] In % of the total flash-induced change in the NaCl-washed sample.
[b] Corrected for 20% intact centers.

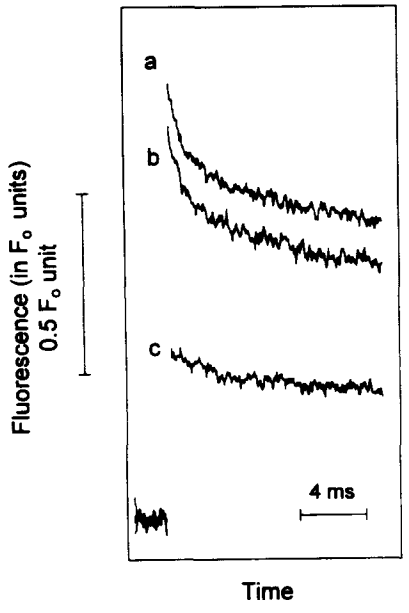

Fig. 1. Effect of Ca^{2+} depletion on fluorescence yield and fluorescence decay kinetics in PS II. Trace a, NaCl-washed PS II membranes (control); trace b, Ca^{2+}-depleted PS II membranes *plus* 20 mM Ca^{2+}; trace c, PS II membranes depleted of Ca^{2+} by washing with NaCl/EGTA. The traces shown represent the sum of 10 individual traces obtained with a flash spacing of 20 s. The PS II membranes were suspended in 25 mM MES-NaOH (pH 6.5), 20 mM NaCl and 0.3 M sucrose (a) with 10 mM EGTA (b, c).

reported for intact thylakoids [21] but within the range of the longer half-times observed in more resolved PS II preparations [22,23]. The > 400 ms decay component most likely corresponds to recombination of charges on the reduced PS II acceptor side with the oxidized donor side [24] in those centers where the Q_A^- to Q_B transfer is inhibited. The validity of this assignment of fluorescence yield components in the NaCl-washed samples was demonstrated by the conversion of the two fast phases into the slow phase in the presence of DCMU, which blocks electron transfer between Q_A and Q_B (not shown). The relative contribution of the slow phase in the NaCl-washed samples was somewhat larger than in non-treated BBY membranes (Table 1). This effect is possibly due to some modification of the acceptor side, which affects the Q_A^- to Q_B electron transfer, induced by a structural modification in the absence of the 16 and 23 kDa polypeptides.

As shown in Fig. 1, depletion of Ca^{2+} resulted in a lowering of the amplitude of the fluorescence rise to less than 50% of that observed in NaCl-washed control PS II membranes. The decrease in amplitude is likely to arise from an increased recombination between Q_A^- and $P680^+$ (not resolved in these measurements, since, due to instrumental limitations, the detection of fluorescence starts about 120 μs after the excitation flash [19]) which competes with other reactions of Q_A^-. An increase in charge recombination was proposed to result from inhibition of the electron transfer to $P680^+$ following the removal of Ca^{2+} [9].

Since electron transfer from TyrZ to $P680^+$ still is functional in the absence of Ca^{2+}, as demonstrated by the formation of $TyrZ^+$ (see below), a fraction of Q_A^- cannot back-react with $P680^+$ and must transfer its electron elsewhere. The situation in these centers is analogous to that in normal centers with bound Ca^{2+}. Consequently, the residual Q_A^- should disappear via the same routes, provided Ca^{2+} depletion did not affect the corresponding reactions. Therefore, in the absence of such specific effects the residual fluorescence is expected to show the original three transients of decay with lower absolute amplitudes but with the same relative sizes as observed in the presence of Ca^{2+}.

When the decay kinetics of the flash-induced fluorescence were measured in the absence of Ca^{2+}, the same kinetic components could be detected as in the presence on Ca^{2+}, albeit with smaller amplitudes. However, if corrections are made for the population of centers with Ca^{2+} still bound (about 20% according to activity measurements in the absence of added Ca^{2+}, see also [9]), it can be seen that the 400–500 μs decay transient, representing Q_A^- to Q_B electron transfer, was completely eliminated as a result of Ca^{2+} depletion (Table 1). At the same time the contribution of the slowest phase (> 400 ms; corresponding to recombination with oxidized donor-side components other than $P680^+$) to the total amplitude increased. This is a consequence of the unequal disappearance of the various reaction phases. As explained above, the reduction in amplitude of the induced fluorescence by $P680^+/Q_A^+$ charge recombination should depress the absolute amplitudes of the three fluorescence-decay components in proportion to their original sizes. Since this was not observed but depletion of Ca^{2+} resulted in a selective elimination of only the 400–500 μs decay transient, our results suggests that Ca^{2+} depletion prevented or radically slowed down the electron transfer from Q_A^- to Q_B (in addition to the earlier described inhibition of the reduction of $P680^+$ by the water-oxidizing complex). A reduction of the rate appears to be the more likely alternative, since the presence of a 4 ms transient suggest that some transfer to the plastoquinone pool still occurs.

3.2. Ca^{2+} titration experiments

On readdition of Ca^{2+} to the depleted PS II membranes, the 400 μs fluorescence yield component reappeared (Fig. 1, Table 1). This indicates that the lesion on the acceptor side induced by depletion of Ca^{2+} had been eliminated and that electron transfer from Q_A^- to Q_B functions as in the NaCl-washed control material. Furthermore, the loss of total amplitude in the flash-induced fluorescence was almost fully regained (Fig. 1, Table 1) which indicates that also donor side electron transfer had

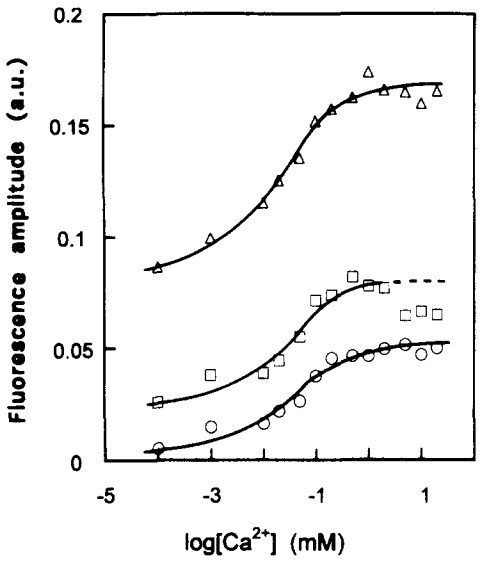

Fig. 2. Dependence of the amplitudes of the fluorescence yield kinetic transients on the concentration of added Ca^{2+}. Ca^{2+}-depleted PS II membranes were suspended in 25 mM MES-NaOH (pH 6.5), 20 mM NaCl and 0.3 M sucrose at the concentrations of Ca^{2+} shown. The symbols represent amplitudes of kinetic transients with the following half-times: ○, 400 μs; □, 4 ms; △, >400 ms. The experimentally observed amplitudes have been corrected for the presence of 20% normal, fully functional centers which are remaining after the Ca^{2+} depletion procedure.

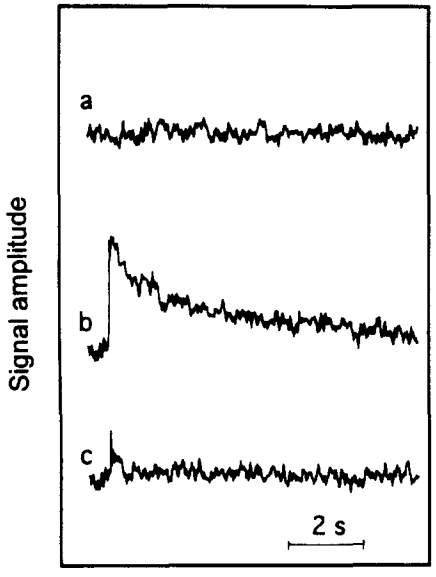

Fig. 3. The effect of Ca^{2+} on $TyrZ^{ox}$ EPR amplitude and reduction kinetics in PS II. Trace a, NaCl-washed PS II membranes; trace b, NaCl/EGTA-treated PS II membranes; trace c, NaCl/EGTA-treated PS II membranes *plus* 20 mM Ca^{2+}. The PS II membranes were suspended in 25 mM MES-NaOH (pH 6.5), 20 mM NaCl and 0.3 M sucrose (trace a) with 10 mM EGTA (b, c). Each trace represents the average of 50 transients (flash spacing 40 s). The traces were obtained at a field setting of 347 mT, close to the low-field maximum in the derivative spectrum shown in Fig. 4A. Instrument settings: microwave frequency, 9.78 GHz; field modulation amplitude, 0.5 mT; microwave power, 20 mW (10 dB); time constant, 20 ms; sample temperature, 293 K.

been restored. The dependence of the amplitudes of the different fluorescence yield components on the concentration of added Ca^{2+} is shown in Fig. 2. All components, including the total amplitude of the fluorescence, appear to be influenced by binding of Ca^{2+} to a site with $K_d = 50$ μM. This value is in fair agreement with values for the dissociation constant for a Ca^{2+}-binding site associated with oxygen-evolution activity [8].

3.3. Donor side

The decrease in fluorescence yield after depletion of Ca^{2+} suggests a more efficient recombination between the reduced acceptor quinone, Q_A^-, and $P680^+$. A facilitated charge recombination may be caused by a retardation of electron transfer from the secondary donor, TyrZ, to $P680^+$

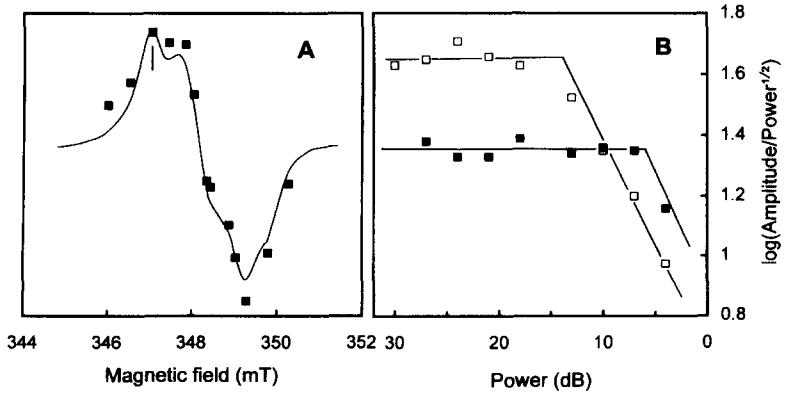

Fig. 4. EPR properties of the flash-induced EPR component in Ca^{2+}-depleted PS II membranes. Panel A, EPR spectrum of the transient component. The amplitude was measured at the field positions indicated by the symbols. Instrument settings as in Fig. 3. For comparison the EPR spectrum of $TyrD^{ox}$ (normalized to approximately the same amplitude) is shown as the full line, measured with EPR settings as for the transient component but microwave power 0.4 mW (27 dB). The bar represents the field position for the kinetic measurements in Fig. 3. Panel B, power saturation properties of transient EPR component (■) and $TyrD^{ox}$ (□). Measuring conditions (except for microwave power) as given in Fig. 3.

Table 2
Light-induced formation of EPR signals from donor-side components

	EPR signal amplitude (% of maximal)	
	Flash-induced $TyrZ^{ox}$ [a]	Split radical signal [b]
NaCl-washed	0	0
Ca^{2+}-depleted		
1 flash	25	26
continuous light	45	41
Ca^{2+}-depleted + PPBQ		
1 flash	33	44
continuous light	45	100

[a] Measured at room temperature as described in Fig. 3. The amplitude is given as percent of PS II centers and is estimated by comparison with the amplitude of the $TyrD^{ox}$ EPR signal (Signal II_{slow}) which is present in all centers (1 equivalent/PS II).
[b] The amplitude of the split radical signal was measured at 10 K as described in Fig. 5.

or an accumulation of $TyrZ^{ox}$ due to inefficient transfer from the oxygen-evolving complex. To investigate the latter possibility, room temperature EPR studies were made of the reduction of $TyrZ^{ox}$ after a laser flash. In NaCl-washed PS II in the presence of Ca^{2+}, no light-induced, time-dependent EPR signals were seen (Fig. 3), suggesting that the reduction of $TyrZ^{ox}$ is rapid, in agreement with other studies, and complete within the response time of the instrument. Ca^{2+} depletion led to the light-induced formation of an EPR signal, with the same line shape as that of the dark-stable EPR signal from $TyrD^{ox}$ (Signal II) and formed on top of this, implying that the signal arises from oxidation of TyrZ (Fig. 4A). This identification was also corroborated by microwave saturation studies (see below). The appearance of the transient EPR signal was almost completely suppressed by the readdition of Ca^{2+}.

The transient EPR signal decayed biphasically with half-times of 600 ms and 5 s, following excitation by single flashes. It had a total amplitude corresponding to about 25% of that of $TyrD^{ox}$ (Table 2) (0.25 equivalents with $TyrD^{ox}$ corresponding to 1 equivalent). With 0.5 mM PPBQ present as an electron acceptor, this value increases to about 35% (Table 2) at the same time as the half-times of the two decay transients increased to 2.3 and 15 s, respectively (not shown). In continuous light the light-induced signal accumulated to an amplitude of about 50% of that of the $TyrD^{ox}$ signal, both in the presence and absence of PPBQ.

Microwave power saturation studies can be used to discriminate between the $TyrZ^{ox}$ and $TyrD^{ox}$ EPR signals, since the magnetic relaxation properties of the two radicals are influenced by the their different environments, mainly differences in magnetic interaction with the manganese center. These differences cause the EPR signal from $TyrD^{ox}$ to saturate more easily than that of $TyrZ^{ox}$. Saturation studies gave a $P_{1/2}$ (see e.g. [25]) of at least 50 mW at room temperature for the light-induced Tyr^{ox} signal versus 8–10 mW for the dark-stable $TyrD^{ox}$ signal (Fig. 4B). Since the measuring microwave power in Figs. 3 and 4A, 20 mW (10 dB), causes partial saturation of the signal from $TyrD^{ox}$, a change in the relaxation properties of this tyrosine and its EPR amplitude, induced by a change in the oxidation state of the manganese cluster after a flash might have influenced the measurements. However, also at a microwave power of 0.4 mW (27 dB), which is non-saturating for $TyrD^{ox}$ (Fig. 4B), we observed a flash-induced signal with an intensity approximately 25% of that of $TyrD^{ox}$. Thus, our results suggest that the light-induced signal arises from $TyrZ^{ox}$ rather than being caused by a light-induced change in the relaxation properties of $TyrD^{ox}$.

In repetitive flash experiments there is a risk that the interval between the flashes is made too short to allow for complete recovery of the initial state. In the case of PS II, accumulation of Q_A^- would lead to a successive decrease in the amplitude of the light-induced EPR signal. A similar decrease in the amplitude of the light-induced transient might be seen if electron transfer from the manganese site would be rate limiting, since $TyrZ^{ox}$ would not be completely re-reduced between the flashes. This would eventually lead to an accumulation of $TyrZ^{ox}$ (and the Ca^{2+}-depleted S_3 state, which cannot be further oxidized).

However, the same light-induced, transient EPR signal, in terms of both amplitude and decay kinetics, which was observed with repetitive laser flashes, was found in 'first-flash' experiments when a single flash was given to Ca^{2+}-depleted material which had been dark-adapted for up to 10 min. This implies that there is enough time between the flashes in the repetitive-flash experiment (40 s between flashes) for the centers to return to the same stable, dark-adapted state (i.e. the dark-stable S'_2 state) between the flashes. In experiments, where the interval between the flashes was reduced to 1 s (i.e., before full disappearance of the light-induced EPR transient), the yield of the light-induced EPR signal was significantly reduced on the second and ensuing flashes due to accumulation of the transient EPR signal (not shown).

The formation of the modified S_3 state was monitored by quickly freezing (within a few seconds) dark-adapted samples in EPR tubes after exposure to flashes of light. The stability of the resulting split radical EPR signal at $g = 2$, width 13 mT, was determined by incubating the flash-illuminated samples in the dark for various times before freezing. At room temperature, the half-time for the disappearance of the split radical signal was 5–7 s in the absence of PPBQ, which increased to about 15 s with PPBQ present (Fig. 5). Thus, the 40 s flash interval was sufficient to allow for most of the centers to relax to the original state between the flashes.

The amount of split radical signal of the S'_3 state after one flash was about 25% of that seen after illumination with strong continuous light in the presence of the electron acceptor PPBQ. As with the transient $TyrZ^{ox}$ EPR signal, PPBQ increased this amount significantly (Table 2). The effect is probably due to the ability of PPBQ to function as

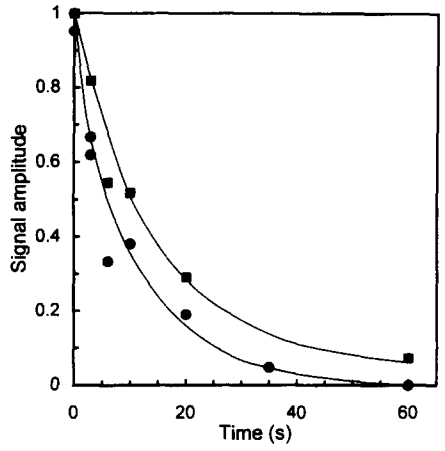

Fig. 5. Lifetime of the modified S'_3 state. Ca^{2+}-depleted PS II membranes in EPR tubes were exposed to 4 flashes (3 Hz) from a frequency-doubled Nd-YAG laser at room temperature (293 K) and after a defined incubation time quickly frozen in a solid CO_2-ethanol bath. The relative amount of the S'_3 state was determined from the amplitude (high-field peak, see [10]) of the EPR signal of the corresponding radical. Symbols: ●, without electron acceptor present; ■, in the presence of 1 mM PPBQ. Measurement conditions: microwave frequency, 9.24 GHz; microwave power, 20 mW; field modulation amplitude, 2 mT; temperature, 10 K.

an electron acceptor, which should lead to a stabilization of charge separation and oxidation of the donor side.

Readdition of Ca^{2+} to the depleted membranes led to an almost complete disappearance of the slow kinetics of $TyrZ^{ox}$ reduction (Fig. 3), conceivably by restoring rapid electron donation to the tyrosine from the oxygen-evolving complex. A small slow component, with an amplitude less than 15% of the original, remained after readdition of Ca^{2+} (Fig. 3), probably as a result of the less than 100% reconstitution, and may represent PS II centers which had been irreversibly damaged during the depletion procedure. As expected, also the split radical signal from the S'_3 state disappeared after readdition of Ca^{2+} (not shown).

3.4. Effects on the acceptor-side non-heme iron

Since our fluorescence yield measurements indicated that Ca^{2+} depletion affects electron transfer events on the PS II acceptor side, which may involve the FeQ center, the EPR signal of the acceptor iron after oxidation with ferricyanide was investigated following the removal of Ca^{2+}. It was found that the EPR signal was identical with that in the Ca^{2+} sufficient centers (Fig. 6), with g-values of 8.05 and 5.61. However, a slight decrease in the amplitude of the signal could be observed, suggesting that the redox properties or the accessibility of the center might have been slightly changed by the removal of Ca^{2+}. No significant modification of the shape or yield of the EPR signal from the reduced acceptor, FeQ_A^-, could be detected in Ca^{2+}-depleted PS II membranes treated with 50 mM formate to enhance the signal (not shown).

3.5. Chemical modification with DEPC

Ca^{2+} depletion has been suggested to alter or introduce an interaction between the manganese cluster and a histidine residue. Thus, modification of this histidine might induce further effects on the partially inhibited electron transfer of Ca^{2+}-depleted PS II. The effect of chemical modification of PS II membranes with the histidine modifier DEPC on the fluorescence kinetics is shown in Fig. 7. Chemical modification of NaCl-washed membranes led to the disappearance of mainly the 400 μs and 4 ms decay transients of Q_A^- to Q_B and Q_A^- to plastoquinone, respectively, with just a slight (about 15%) decrease in the total amplitude (Fig. 7A). Depletion of Ca^{2+} followed by chemical modification resulted in both the loss of the rapid fluorescence yield kinetics and a decrease in the amplitude of the total fluorescence yield of which only the amplitude could be partly reversed by the addition of Ca^{2+} (Fig. 7B).

EPR studies of Ca^{2+}-depleted, DEPC-modified PS II membranes showed no significant differences with regard

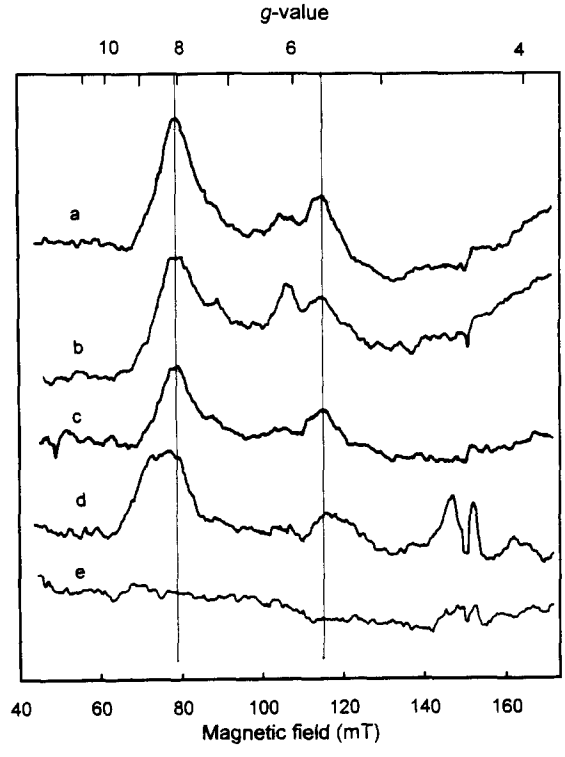

Fig. 6. EPR spectra of the oxidized acceptor-side iron in PS II. PS II membranes were treated for 30 min with 5 mM $K_3Fe(CN)_6$ in darkness before freezing at 77 K. The spectra represent differences between spectra taken before and after photoreduction of the acceptor iron by illumination at 200 K. Trace a, control PS II membranes; trace b, NaCl-washed PS II membranes; trace c, NaCl/EGTA-treated; trace d, NaCl-washed modified with DEPC; trace e, NaCl/EGTA-treated modified with DEPC. The vertical lines are included to help in the comparison of the peak positions of the acceptor iron spectrum. Measurement conditions: microwave frequency, 9.24 GHz; microwave power, 32 mW; field modulation amplitude, 2 mT; temperature, 5 K.

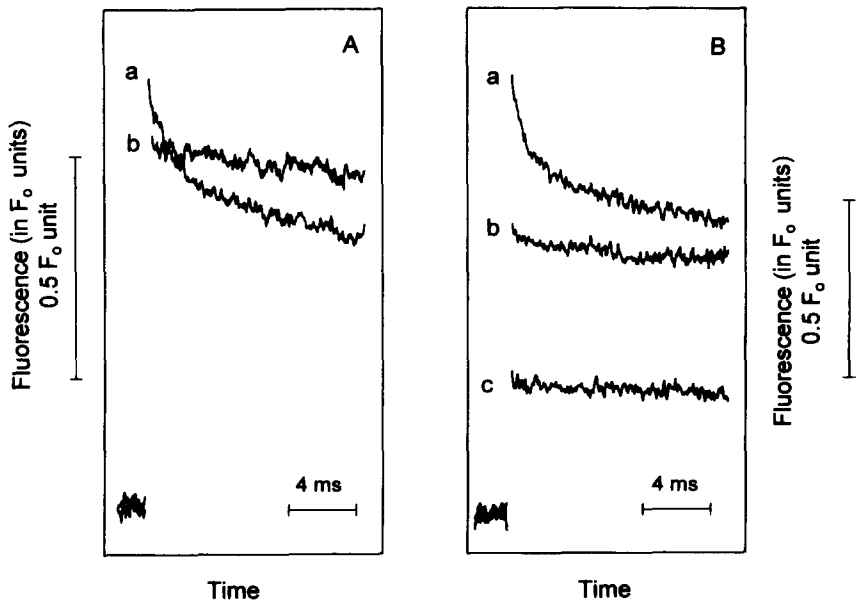

Fig. 7. Effect of chemical modification of histidine with DEPC on flash-induced fluorescence kinetics in PS II membranes. (A) Trace a, NaCl-washed control; trace b, treated with DEPC. (B) Trace a, NaCl-washed control; trace b, Ca^{2+}-depleted and DEPC-modified, reconstituted with 20 mM Ca^{2+}; trace c, Ca^{2+}-depleted and DEPC-modified. Experimental conditions as in Fig. 1.

to the $TyrZ^{ox}$ kinetics when compared to unmodified, Ca^{2+}-depleted membranes, but the formation of the split radical of the S'_3 state was suppressed (about 50%) after a flash (not shown).

To investigate whether the chemical modification of histidine did cause any EPR-detectable changes in the environment of the acceptor-side iron, low-temperature EPR spectra were taken after treatment with 50 mM formate and illumination at 77 K. No significant effects were detected in yield or shape in the FeQ_A^- EPR signal after DEPC-treatment of control NaCl-washed samples or after Ca^{2+}-depleted (not shown). Treatment of the DEPC-modified PS II membranes with ferricyanide resulted in the oxidation of the acceptor-side iron (Fig. 6). Interestingly, the lines of the EPR signal showed a significant shift compared to the positions in unmodified centers with new g-values of 8.37 and 5.51, respectively.

In contrast, ferricyanide treatment of Ca^{2+}-depleted membranes, modified with DEPC, did not result in any EPR signals typical of oxidation of acceptor-side iron (Fig. 6), indicating an increase in midpoint potential of the iron or a substantial change in the accessibility of the acceptor side for the oxidant.

4. Discussion

The inhibition of oxygen evolution catalyzed by PS II which is observed after removal of Ca^{2+} by washing in Ca^{2+}-complexing media has been suggested to be caused by an inhibition of further oxidation of the S'_3 state [10–12]. This effect has recently been suggested to result from the inability to photo-oxidize TyrZ once the S'_3 state has formed, at least following one type of Ca^{2+}-depletion procedure [9]. However, our experiments show that the effects of Ca^{2+} depletion on PS II electron transfer are more complex with effects on both donor and acceptor sides. One effect of Ca^{2+} depletion observed here and which is indicated by the appearance of an EPR signal from $TyrZ^{ox}$ and its slow reduction, is that the rate of electron transfer from the Mn-cluster to tyrosine has been slowed down, with a change in time constant from about 100 μs to the seconds range. Thus, we observe that photooxidation of TyrZ may still occur after Ca^{2+}-depletion. This is seemingly in conflict with results by Boussac et al. [9], who could not observe an EPR signal from oxidized TyrZ. However, this apparent contradiction is neatly explained if oxidation of TyrZ can occur in the S_2 but not in the S_3 state. In the case of Boussac et al., the short interval between the flashes (4 s) would not have allowed efficient relaxation back to the S_2 state but would instead have led to the accumulation of the modified S_3 state as claimed. On the other hand, the long interval between the flashes (40 s) in our case allowed the material to return to the S_2 state after each flash. This is reasonable considering the half-times of decay of the light-induced fluorescence (Fig. 1, Table 1) and of the EPR signals of $TyrZ^{ox}$ (Fig. 3) and the modified S_3 state in Ca^{2+}-depleted PS II (Fig. 5). Furthermore, our conclusion is strengthened by the observation that the same reduction kinetics of $TyrZ^{ox}$ was observed also in 'first-flash' experiments with material which had been dark-adapted for several minutes and which is known to reside in the dark-stable S_2 state. In these experiments each sample was given just one flash to

avoid the complication due to possible non-decaying higher S-states. It might be added that evidence of effects on the electron transfer from the manganese after depletion of Ca^{2+} have been found earlier, which indicated that also the S_1 to S_2 transition was inhibited by Ca^{2+} depletion [26].

Another effect of Ca^{2+} depletion on PS II donor side electron transfer is suggested by the decrease in the fluorescence amplitude. This is an indication of an enhancement of the charge recombination between $P680^+$ and Q_A^- which occurs with microseconds kinetics, faster than the resolution of our detecting system. Enhancement of the $P680^+/Q_A^-$ recombination has earlier been observed after Ca^{2+} depletion [9,27]. The increase of the recombination is evidence that the transfer between TyrZ and $P680^+$ has been retarded. In this case, the slower reduction of $P680^+$ is not a consequence of the slow electron transfer between Mn and $TyrZ^{ox}$ and a resulting accumulation of $TyrZ^{ox}$, since, with a spacing between flashes of 20 s, most of the PS II centers have enough time to revert to their original condition, $S'_2ZP680Q_A$, between the flashes. An increase in the μs decay kinetics of $P680^+$, attributed to charge recombination due to donor-side inhibition, was, in fact, noted already after the first flash by Boussac et al. [9] in PS II, depleted of Ca^{2+} and polypeptides.

One striking observation in this study is the pronounced similarity between the behavior of the $TyrZ^{ox}$ EPR signal and the split radical EPR signal of the S'_3 state: (i) Both signals appear reversibly as a result of depletion of Ca^{2+}. (ii) The intensity of the signals seem to respond in a similar manner to different illumination conditions and PPBQ (Table 2). (iii) The slow half-time of $TyrZ^{ox}$ decay and the decay of the S'_3 signal at room temperature are similar and increase similarly after addition of PPBQ.

A difference between the two signals is that a rapid decay transient in S'_3 state signal, corresponding to the rapid phase in the disappearance of the $TyrZ^{ox}$ signal, was not observed. However, such a rapid decay transient would have been beyond the time-resolution of the freeze method (freezing time 1–2 s) used to trap the S'_3 state signal in our experiment and might well have been overlooked.

The parallel behavior of the detectable portion of the S'_3 state signal and the $TyrZ^{ox}$ signal suggest a close connection between the two responsible species. Recently, Hallahan et al. [28] suggested that the split radical signal did not arise from histidine interacting with the manganese cluster, but that the responsible amino acid was $TyrZ^{ox}$. Very recent results from pulsed ENDOR investigations also strongly indicate that the split-radical signal arises from $TyrZ^{ox}$ in PS II membranes depleted of Ca^{2+} by low pH/citrate treatment (D. Britt, University of California at Davis, personal communication). Our results are in full agreement with these studies.

The more efficient charge recombination between $P680^+$ and Q_A^-, which has been found to correspond to about 0.5 equivalents after Ca^{2+} depletion in this type of preparation [9], is to a significant extent responsible for the rather low amount of $TyrZ^{ox}$ (0.2–0.3 equivalents), observed after a flash. Another factor which may contribute to the apparent low yield of $TyrZ^{ox}$ is recombination with reducing equivalents on the acceptor side. This is supported by the observation that both the amplitudes and the decay kinetics of the EPR signals from $TyrZ^{ox}$ and the S_3 state increase significantly after a flash when PPBQ is present to accept electrons from Q_A^- (Table 1).

The observations described above show that Ca^{2+} depletion of PS II membranes results in changes in the electron transfer rates on both the donor and acceptor sides. The retardation of electron transfer on the acceptor side is in agreement with recent observations by Krieger et al. [14], which indicate that depletion of Ca^{2+} raises the midpoint potential of the Q_A/Q_A^- redox couple by 120 mV (however, see [15]). They also suggested that release of Ca^{2+} from a site on the PS II donor side could have affected the acceptor side although the simultaneous release of Ca^{2+} from a site on the acceptor side was not excluded. Other observations indicating that changes at the donor side of PS II can influence the characteristics of acceptor-side components have been reported. For example, when the 33 kDa extrinsic protein is absent from the donor side in PS II, the stability of Q_A^- is increased in both isolated PS II membranes [29] and in intact cyanobacteria [30,31].

In the present work we show that the acceptor-side inhibition, measured from the effect on fluorescence kinetics, is dependent on the occupancy of a Ca^{2+}-binding site with a K_d of 50 μM. This value is in very good agreement with affinity determinations for a site which becomes accessible after removal of the 16 and 23 kDa extrinsic proteins and which is considered to be necessary for the functionality of the PS II donor side (see [1] for a recent review on Ca^{2+} binding). Also, the loss in amplitude of the flash-induced fluorescence, which reflects $P680^+/Q_A^-$ charge recombination and which is likely to be dependent on donor-side electron transfer rates, seems to be effected by the occupancy of a binding site with the same dissociation constant. A very recent study of Ca^{2+} binding in PS II using ^{45}Ca [32] showed that the binding of a single Ca^{2+} ion with a K_d of 60 μM could reactivate water-oxidation in Ca^{2+}-depleted PS II centers with the 16 and 23 kDa proteins present. A similar dissociation constant for a single exchangeable Ca^{2+} ion was found also in polypeptide-depleted PS II. Taken together with the effects of Ca^{2+} depletion and reactivation presented in the present work, these results strongly suggest that a single Ca^{2+} ion controls electron transfer on both donor and acceptor sides in PS II. If the binding of the ion takes place on the donor side of the thylakoid membrane, the effects of binding on this side might be mediated across the membrane to the acceptor side with the transmembrane helices acting as levers. It is not possible at this stage to give a closer description of the type of structural changes which may

occur on the acceptor side as a result of Ca^{2+} depletion and which lead to the inhibition of the electron transfer between the Q_A and Q_B sites. The absence of large effects on the EPR signal of the oxidized acceptor-side iron, however, indicates that the environment around the iron is largely unaffected, suggesting that any structural changes on the acceptor side may be subtle in nature.

Depletion of Ca^{2+} has been suggested to alter or introduce an interaction between the manganese cluster in PS II and a histidine residue. Thus, chemical modification of this residue might be expected to be detectable as an effect on the fluorescence yield. The slight decrease in total fluorescence amplitude, which follows after treatment with DEPC (Fig. 7A), an indication of a retardation of donor-side electron transfer, may be taken as evidence of a modification of a donor-side histidine. The suppressed formation of the split S'_3 state EPR signal after DEPC modification agrees with earlier observations [33] and supports a donor-side modification. The modification does not appear to have influenced the binding of Ca^{2+} significantly since, when the chemical modification was followed by Ca^{2+} depletion, there was a further decrease in the fluorescence amplitude which could be reversed by readdition of Ca^{2+} (Fig. 7B) as in unmodified membranes. Instead, the main effect of modification of histidine with DEPC is an abolishment of the Q_A^- to Q_B electron transfer which is visible as the disappearance of the 400 μs fluorescence kinetics (Fig. 7). In this case, the inhibition may be related to the observed small change in the EPR properties of the non-heme iron after the modification of histidine (cf. Fig. 6a and d). The effect on the iron appears to be particularly prominent in connection with depletion of Ca^{2+}, since in this case treatment with ferricyanide did not result in an EPR signal from Fe(III) (Fig. 6e). The absence of an Fe(III) EPR signal is not likely due to loss of iron from the acceptor side since a normal EPR signal from FeQ_A^- was formed on photoreduction of the acceptor side in the presence of formate (see also [33]), but could result from an increase in reduction potential of the iron or a change in accessibility of the acceptor side for the oxidant.

We cannot at this stage tell where the modification-susceptible histidine, influencing the acceptor-side electron transport, is located. The effects of DEPC on the EPR properties of the oxidized acceptor-side iron may be taken to support a localization to the PS II acceptor side. A mutation in one of the histidine ligands to the acceptor-side iron resulted in impaired Q_A^- oxidation [19], similar to what is observed here in the absence of Ca^{2+}. It is also possible that, after Ca^{2+} depletion, DEPC binds to occupy the Q_B site, which would explain not only the inhibition of Q_A^- to Q_B electron transfer but also the inhibited oxidation of the non-heme iron which occurs via the Q_B site [34]. On the other hand, the effect of Ca^{2+} depletion on fluorescence induction together with other observations [14,29–31] suggest that structural changes on the donor side in PS II may have significant influence on electron-transfer properties on the acceptor side. Therefore, we cannot exclude that modification by DEPC of a donor-side histidine is responsible for inhibition of the electron transfer between Q_A^- and Q_B. The enhanced effect of DEPC in the absence of Ca^{2+}, as seen in EPR by the inability to oxidize the acceptor-side iron, might indicate that Ca^{2+} depletion induces a conformational change which makes additional residues accessible to the modifying agent.

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