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Comparison of chloride-depleted and calcium-depleted PSII: the midpoint potential of Q_A and susceptibility to photodamage

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Abstract

Photosystem II has been studied in membranes in which O_2 evolution was inhibited by depletion of either chloride or calcium ions. It has been shown earlier [Krieger, A. and Weis, E. (1992) Photosynthetica 27, 89–98] that depletion of calcium ions results in a 150-mV up-shift of the midpoint redox potential (Em) of Q_A/Q_A^- (the protein-bound plastoquinone which acts as an electron acceptor). Here it is shown that chloride depletion has no effect on the Em of Q_A/Q_A^-. It is also demonstrated that chloride-depleted PSII is more susceptible than Ca^{2+}-depleted PSII to damage by light. This extra susceptibility to light in Cl^--depleted PSII is eliminated when the artificial electron acceptor DCPIP is present during illumination. These observations are consistent with the hypothesis that the up-shifted Em of Q_A/Q_A^- in Ca^{2+}-depleted PSII results in a protection of the reaction centre from damage by light by changing the dominant charge recombination pathway to one which does not involve formation of the $P680^+Ph^-$ radical pair, the P680 triplet and singlet oxygen [Johnson, G.N., Rutherford, A.W. and Krieger, A. (1995) Biochim. Biophys. Acta 1229, 202–207].

Keywords: Photosynthesis; Photosystem II; Photoinhibition; Q_A; Redox titration

1. Introduction

The photoinactivation of photosystem II (PSII) has been extensively studied when PSII is exposed to light intensities higher than those required for saturating photosynthetic electron flow (reviewed in Refs. [1–3]). It has been observed that, upon inhibition of oxygen evolution, PSII becomes particularly vulnerable to light-induced damage [4–12]. A variety of different treatments which inhibit oxygen evolution have been reported to result in an increased susceptibility of PSII to damage by light: e.g., Cl^--depletion [4,5,7–11], washing with NH_2OH [5,8,9] or Tris [4,10]. It was found that illumination of PSII lacking O_2 evolution results in inhibition of electron transport through PSII (measured using artificial electron donors), and this leads to a rapid turnover of the D1 reaction centre protein. It was suggested that the light-induced degradation of the D1 protein is triggered by the oxidation of the primary electron donor chlorophyll, P680, and/or the secondary electron donor, a tyrosine (TyrZ) [4,7,9,10,12]. Both of these electron donors are known to have extended lifetimes

Abbreviations: Chl, chlorophyll; DCPIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; Em, midpoint redox potential; MES, 4-morpholineethanesulfonic acid; P680, the photooxidizable chlorophyll in PSII; Ph, pheophytin; PSII, photosystem II; Q_A, the first quinone acting as an electron acceptor in PSII; Tris, Tris(hydroxymethyl)aminomethane; Tyr, tyrosine.

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when electron donation from water is inhibited (reviewed in Ref. [13]). The extended lifetime of $P680^+$ (and possibly $TyrZ^{\cdot}$ seems to lead to oxidation of other reaction centre components, a monomeric chlorophyll Chl, and a carotenoid; the oxidation of these components may be a symptom or a trigger for photodamage to the reaction centre [6,8,9].

The release of Ca^{2+} from PSII results in inhibition of O_2 evolution (reviewed in Refs. [14–16]). It has been suggested that this occurs when high concentrations of protons are generated in the lumen under conditions of strong illumination [17]. It has also been suggested that the inhibited PSII resulting from Ca^{2+} release may represent a state in which excess light energy can be dissipated as heat via charge recombination [17].

PSII lacking Ca^{2+} was shown to have a Em value for the Q_A/Q_A^- redox couple which was 150 mV higher than in active PSII [17,18]. It was pointed out that such different Em values for Q_A/Q_A^- could determine the charge recombination pathway for $P680^+Q_A^-$ [19]. When low potential Q_A is present, charge recombination probably occurs via $P680^+Ph^-$ (and hence P680 triplet), while this route is thermodynamically unfavourable when the high potential form of Q_A is present [19]. This modulation of the midpoint potential of the Em of Q_A/Q_A^- by Ca^{2+} occurs during photoactivation of PSII [19], the process by which PSII assembles the Mn cluster which acts as the charge accumulation site and probably the active site for oxygen evolution (reviewed in Refs. [15,20]). The regulation of the electron transfer and charge recombination pathways, which results from the Ca^{2+}-dependent change in the Em of Q_A/Q_A^-, is thought to represent an important protective mechanism during photoactivation [19].

As described above, Cl^--depleted PSII membranes have already been studied in terms of their susceptibility to light. Few such studies [11] have been done on Ca^{2+}-depleted PSII although the lesions on the electron donor side have been found to be comparable to those in Cl^--depleted PSII [16,21,22]. In this study we have measured the Em of Q_A/Q_A^- in Ca^{2+} and Cl^--depleted PSII membranes under comparable conditions and have directly compared their susceptibility to damage by light. The results indicate the existence of a specific Ca^{2+}-dependent protective mechanism.

2. Materials and methods

PSII-enriched membrane fragments from spinach were prepared essentially as described by Berthold et al. [23] with modification as described by Johnson et al. [24]. The activity of these samples was about 500 μmol O_2/mg chl · h. Ca^{2+}-depletion was performed by incubation of PSII samples at room temperature for 5 min in room light (10–12 μmol quanta m^{-2} s^{-1}) in a buffer containing 300 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 25 mM succinic acid (pH 4.5). The residual activity was very low (20–50 μmol O_2/mg chl · h). Samples were then centrifuged and washed in a medium containing 300 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 30 mM MES (pH 6.5). By re-addition of $CaCl_2$, 70–80% of the activity of a control sample was obtained. Cl^--depletion was done by alkaline pH-treatment in room light as described by Homann [21], sucrose 'Superpur' was used to reduce the chloride contamination. PSII samples were incubated for 30 s at pH 10 (50 μmol O_2/mg chl · h measured at pH 6.5). By re-addition of NaCl, 80% of the activity of a control sample was obtained in these samples.

NH_2OH-treatment was done by incubating PSII samples in 5 mM NH_2OH in a buffer containing 400 mM sucrose, 15 mM NaCl and 50 mM MES (pH 6.5) followed by two washes in buffer without NH_2OH.

For redox titrations, samples at a concentration of approx. 50 μg Chl ml^{-1} were put into a home-built cuvette and maintained at all times under argon. The redox potential was measured at 20°C by means of a platinum electrode, with a silver/silver chloride electrode as reference electrode and connected to a Knick pH/millivolt meter. Measured redox potentials were normalized to the standard hydrogen electrode, calibrating the electrode using saturated quinhydrone (potential = 286 mV at pH 6.5, 25°C). Reductive titrations were performed by the gradual addition of sodium dithionite (in 0.5 M MES, pH 6.5), oxidative titrations by addition of potassium ferricyanide. No redox mediators were used because they have been shown to influence redox titrations of Q_A in active PSII [18]. Fluorescence was measured through a side window of the cuvette using a PAM 101 fluorimeter (Walz, Effeltrich, Germany). Fluorescence was measured using the weak measuring light of the PAM fluorimeter set to 1.6 kHz, as described previously

[17]. Redox titrations were performed in a buffer containing 300 mM sucrose, 25 mM Na_2SO_4 and 25 mM MES (pH 6.5).

As a measurement of PSII activity, the electron transport from DPC to DCPIP was measured at 620 nm in a Shimadzu UV210 spectrometer equipped with side illumination using saturating light (4000 μE). The concentration of chlorophyll concentration was 5 μg/ml for all samples. The assay medium consisted of 300 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 25 mM MES (pH 6.5), 35 μM DCPIP and 1 mM DPC. For measurements with Cl^--depleted samples 25 mM Na_2SO_4 was used instead of KCl and $MgCl_2$. In Ca^{2+}-depleted samples no change in activity was observed when $MgCl_2$ was replaced by Na_2SO_4.

3. Results

Inhibition of the water-splitting complex by Ca^{2+}-depletion leads to an up-shift of the midpoint potential of the redox couple Q_A/Q_A^- from about -80 mV to $+64$ mV [18]. Fig. 1 shows redox titrations of Q_A/Q_A^- measuring the chlorophyll fluorescence yield in PSII-enriched membrane fragments from spinach.

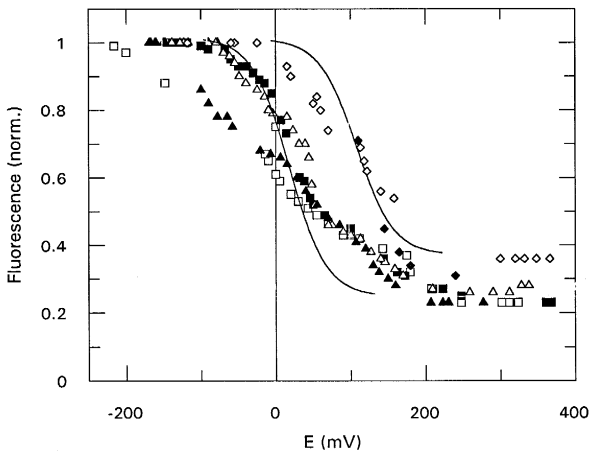

Fig. 1. Redox titrations of chlorophyll fluorescence in active (triangles), Cl^--depleted (squares) and Ca^{2+}-depleted (diamonds) PSII-enriched membrane fragments. The treated samples showed no remaining activity of O_2 evolution. Closed symbols indicate oxidative, open symbols reductive titrations. Na_2SO_4 was used as electrolyte. The curves shown represent one-electron Nernst curves with Em values of $+10$ mV and $+120$ mV. No redox mediators were used in these titrations (see Ref. [18]).

Titrations are shown of PSII which was active in O_2 evolution (triangles) or inactivated by depletion of Ca^{2+} (diamonds) or Cl^- (squares). In Cl^--depleted and active samples the same midpoint potential, about 10 mV, was obtained, while after Ca^{2+}-depletion, the fluorescence titrated with a potential which was 100 mV more positive.

These measurements had to be performed in chloride-free media because Cl^--depleted PSII reactivates easily if any external Cl^- is present. Na_2SO_4 was used instead of KCl as electrolyte. Since sodium sulfate is known to cause significant diffusion potentials, the actual values for the midpoint potential should be regarded with caution. An additional problem due to working under these conditions is that large hysteresis effects were observed and the data do not fit well to theoretical Nernst curves. Nevertheless, it is obvious that the potential dependence of the yield of variable fluorescence is almost identical in samples which are active in O_2 evolution and in those inhibited by Cl^--depletion. In contrast, in samples which are inhibited by Ca^{2+}-depletion, the fluorescence titrates with a more positive midpoint potential as reported earlier [17,18].

The sensitivity of Ca^{2+} and Cl^--depleted PSII to light was compared in samples which were inhibited in their oxygen evolution prior to the photoinhibitory treatment. The electron transfer to DCPIP in the presence of DPC, an artificial electron donor, was measured to monitor electron transfer in PSII. This was done in two different types of experiment. First, in samples which were treated with NH_2OH (to remove the manganese cluster) after photoinhibitory treatment and prior to activity measurements (Fig. 2A). Second, in samples in which DCPIP reduction was measured directly after the photoinhibitory treatment without specific removal of the manganese cluster (Fig. 2B). The difference between these two types of measurement is only that after NH_2OH washing the activity measurement is expected to reflect essentially electron donation from DPC while in the second approach electron donation occurs from water in those centres where the enzyme is functional. Very similar results were obtained in both cases. Fig. 2A and B shows that the preillumination treatment resulted in inhibition of electron transfer in the samples with non-functional oxygen evolution. Fig. 2A and B shows that, Cl^--depletion (filled cir-

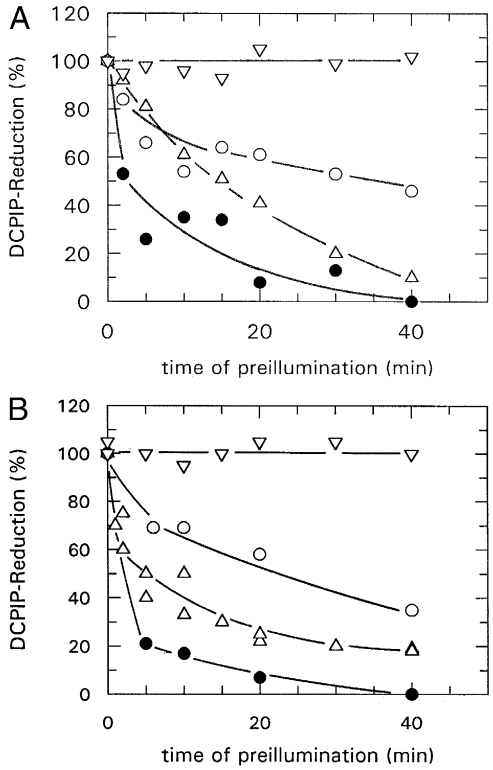

Fig. 2. Photoinactivation of Cl^-- (filled circles) and Ca^{2+}-depleted (open circles) PSII-enriched membrane fragments, in which oxygen evolution was completely inhibited. The samples were incubated with white light (600 μmol quanta m^{-2} s^{-1}) at room temperature. The rate of DCPIP reduction was measured in the presence of 1 mM DPC as electron donor in centres where water oxidation is inhibited. As controls, photoinactivation of active (inverted triangles) and NH_2OH-washed samples (triangles) are shown. (A) Samples were treated with NH_2OH to remove the Mn cluster immediately after the photoinhibitory treatment and prior to measuring the activity. (B) The activity was measured directly without specific removal of the Mn cluster. In (B) the activity of the different samples prior to photoinhibition were the following: control (active) 810 μmol $DCPIPH_2$/mg Chl h^{-1}, Ca^{2+}-depleted 140 μmol, Cl^--depleted samples 400 μmol and NH_2OH-washed 259 μmol $DCPIPH_2$/mg Chl h^{-1}. The different activities in the inhibited samples may be related to access of DPC and/or to rate limitations on the electron acceptor side. The activities in the absence of DPC was less than 5% for Ca^{2+}-depleted, Cl^--depleted and NH_2OH-washed PSII.

cles) leads to a much higher amount of photoinhibition than Ca^{2+}-depletion (open circles). For comparison, photoinhibition of NH_2OH-washed PSII-enriched membrane fragments is shown (triangles). NH_2OH treatment inhibits oxygen evolution by releasing Ca^{2+} and Mn. Such samples are known to be very vulnerable to light [5,8,9]. They were found to be less sensitive towards light than Cl^--depleted samples but more sensitive than Ca^{2+}-depleted samples. NH_2OH treatment, which inhibits O_2 evolution by Ca^{2+} and Mn-release, results in the same up-shift of the midpoint redox potential of Q_A as seen after Ca^{2+}-depletion [17,18]. The illumination treatment used to photoinhibit the Ca^{2+}- and Cl^--depleted PSII in Fig. 2 had little effect on samples which were

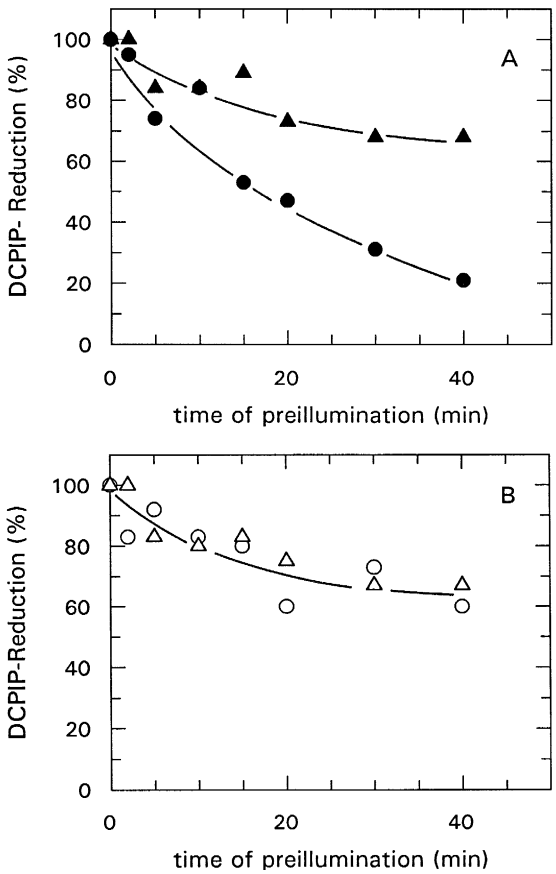

Fig. 3. Effects of the presence of an electron acceptor during photoinactivation of Cl^-- and Ca^{2+}-depleted PSII-enriched membrane fragments. The photoinhibitory treatment was performed as described for Fig. 2. 10 μM DCPIP was added prior to photoinhibitory treatment and re-added after each 10 min of illumination. A: Cl^--depleted sample, filled triangles: DCPIP present, filled circles: no DCPIP during photoinactivation, B: Ca^{2+}-depleted sample, open triangles: DCPIP present, open circles, no DCPIP during photoinactivation. Activity was measured as in Fig. 2B: without prior removal of the Mn cluster. Activity of the different samples prior to photoinhibition: Ca^{2+}-depleted 190 μmol, Cl^--depleted samples 360 μmol $DCPIPH_2$/mg Chl·h.

active in O_2 evolution; the O_2 evolution was lost to a maximum of 10%, while electron transfer through the reaction centre, using DPC as an electron donor, was unaffected (Fig. 2A and B, inverted triangles).

Fig. 3 shows the influence of the presence of an electron acceptor during the photoinhibitory treatment in Ca^{2+}- and Cl^--depleted PSII. It should be noted that a different preparation of PSII-enriched membrane fragments was used, so that the amount of photoinhibition reached by the same treatment was less than shown in Fig. 2. After Cl^--depletion, the addition of DCPIP during the photoinhibitory treatment protected the sample to a large extent. In Ca^{2+}-depleted samples, photoinhibition was independent of the presence or absence of DCPIP. Interestingly, the same amount of photoinhibition was found in Cl^--depleted samples, illuminated in the presence of DCPIP as in the Ca^{2+}-depleted sample. In this experiment we re-added 10 μM DCPIP every 10 min to obtain the optimal protective effect. The addition of DCPIP only at the beginning of the 40 min photoinhibitory treatment resulted in 75% photodamage, while when DCPIP was added twice photodamage occurred to 47% and with three DCPIP additions 32% photodamage occurred (not shown).

4. Discussion

The data presented in this paper show that inhibition of O_2 evolution by depletion of Cl^- does not effect the Em of Q_A/Q_A^- (Fig. 1). This is in marked contrast to the case in which O_2 evolution is inhibited by depletion of Ca^{2+} where the Em of Q_A/Q_A^- is up-shifted by approx. 150 mV [18].

The results also show that Cl^--depleted PSII is more susceptible to photodamage than is Ca^{2+}-depleted PSII. Photodamage in Cl^--depleted PSII is significantly diminished when the artificial electron acceptor DCPIP is present during the illumination treatment. In contrast, the presence of the electron acceptor did not affect the extent of photodamage seen in Ca^{2+}-depleted PSII. Interestingly, when protected by the presence of DCPIP, the extent and the time course of the photodamage occurring in Cl^--depleted PSII became indistinguishable from that occurring in Ca^{2+}-depleted PSII.

The protective effect provided by DCPIP in Cl^--depleted PSII may seem surprising since it has been suggested that photodamage in such material results from over-oxidation of the electron donor components (see Section 1) and therefore the presence of an efficient electron acceptor might have been expected to exacerbate such damage. This result indicates, however, that the main cause of photodamage in Cl^--depleted PSII is not over-oxidation of the donor side components.

Eckert et al. [10] reported for Tris-treated PSII that the presence of an electron acceptor during photoinhibitory treatment resulted in no increase of the photodamage but that the acceptor did not protect against photodamage. This situation is similar to that seen here with Ca^{2+}-depleted PSII but different from that seen with Cl^--depleted PSII. As discussed below, this is consistent with the idea that treatments which result in the up-shift of the Em of Q_A/Q_A^- (e.g., Ca^{2+}-depletion or washing with Tris) should be less susceptible to charge recombination-mediated photodamage [19].

The possibility that over-reduction of the acceptor side components of PSII is responsible for photodamage in Cl^--depleted material is highly unlikely since electron donation is so limited (see, e.g., Refs. [4,22]). A more likely explanation of the protective effect of DCPIP is that removal of the electrons from the reaction centre diminishes the chances of charge recombination occurring and consequently that events associated with charge recombination are responsible for the photodamage.

The photodamage incurred by PSII under conditions where $P680^+Ph^-$ charge recombination occurs seems to involve P680 triplet formation and thence singlet oxygen generation [25,26] and, indeed, singlet oxygen generation presumably mediated by P680 triplet has been demonstrated in PSII [27]. It has been pointed out that charge recombination of $P680^+ Q_A^-$ and other more stable radical pairs in the PSII reaction centre can occur via the $P680^+Ph^-$ radical pair resulting in P680 triplet formation [28] (see also Refs. [19,29]). Keren et al. [29] suggested that charge recombination, even in the intact enzyme under physiological conditions, might lead to triplet-mediated formation of singlet oxygen which is responsible for protein damage.

Since the inhibition of the donor side in Ca^{2+}-depleted PSII is similar to that in Cl^--depleted PSII, it

might have been expected that the photodamage in both kinds of sample would be comparable. Clearly this is not the case. Ca^{2+}-depleted PSII is much less sensitive to light and is unaffected by the presence of the exogenous electron acceptor. This result may be taken as indicating that the photochemical events associated with photoinhibition in Ca^{2+}-depleted PSII are different from those occurring in Cl^- depleted PSII.

The difference in the Em of Q_A/Q_A^- in the Cl^--depleted and Ca^{2+}-depleted PSII may be related to their different susceptibility to photodamage. We suggested earlier from thermodynamic arguments that the shift of the Em of Q_A/Q_A^-, which is found in Ca^{2+}-depleted PSII, results in charge recombination by a pathway that does not involve formation of $P680^+Ph^-$ radical pair and P680 triplet [19]. From the same argument then, the lack of a shift in the Em of Q_A/Q_A^- in Cl^--depleted PSII leads us to suggest that these centres would be more susceptible to damage since charge recombination can occur via $P680^+Phe^-$ and P680 triplet which can react to form singlet oxygen.

It has been reported that H_2O_2 is formed in Cl^--depleted PSII [30,31] and that this contributes to photodamage [31]. Since peroxide formation was modulated by treatments which specifically affected the electron donor-side, it was suggested that peroxide is formed as a result of partial water oxidation [30,31]. However, it was later shown by mass spectroscopy that, at least in the absence of an exogenous electron acceptor, the peroxide was generated from oxygen reduction rather than water oxidation [32]. The reason for the donor-side modulation of the oxygen reduction remained unexplained. The present results suggest an explanation for these phenomena: namely that peroxide formation may be related to the Em of Q_A/Q_A^-.

A mechanism for peroxide formation involving the direct reduction of O_2 from low potential Q_A^- (the high potential form may be unable to do this) can be envisaged, but this does not explain why Cl^-depleted material is more susceptible than untreated PSII. Alternatively, the peroxide may be formed by reduction of singlet oxygen sensitized by the chlorophyll triplet formed by charge recombination. We have suggested above that charge recombination in Cl^--depleted PSII, which contains low potential Q_A/Q_A^-, would lead to P680 chlorophyll triplet and thence singlet oxygen formation. Reduction of singlet oxygen to form peroxide could occur from several reduced or photoreduced components of PSII. From the literature it seems reasonable to suggest that such reactions can occur upon illumination of PSII membranes. It is known that singlet oxygen is more easily reduced than triplet oxygen [33], that reduction of singlet oxygen to form superoxide [34] and peroxide [35] can occur in biological systems, and that superoxide reactions with metal ions can lead to peroxide formation [33].

As mentioned above, when the high potential form of Q_A is present, charge recombination is thought to occur via a route which does not involve formation of the $P680^+Ph^-$ radical pair [19]. Whether this charge recombination is direct via a tunnelling reaction or occurs via other electron carriers is not clear. However, the recent observations that the high potential form of Q_A is present in PSII prior to photoactivation [19] and that cytochrome *b*559 acts as an electron acceptor in such material [36] seems to favour a recombination pathway involving cytochrome b559 under these conditions. A role for cyt *b*559 in photoactivation was hypothesised earlier [37] and a correlation between its redox form and the presence of Ca^{2+} has been reported [38]. A role for cyt *b*559 as part of an electron transfer cycle around PSII has been suggested several times in the past (e.g., Refs. [6,39] for recent examples). Direct evidence for such a cycle might be worth looking for during photoactivation and/or in Ca^{2+}-depleted PSII.

Although Ca^{2+}-depleted PSII is less susceptible to photodamage than is Cl^--depleted PSII, it is nevertheless more sensitive to light than is PSII with functional oxygen evolution. The molecular mechanism of photodamage in Ca^{2+}-depleted PSII is not clear. From previous work we know some of the details of the donor-side events occurring upon illumination of Ca^{2+}-depleted PSII [16,22]. On the first turnover from the dark-adapted S1 state, the Mn cluster is oxidized forming the S2 state. On the second turnover an organic free radical is formed with a high quantum yield [40]. This state is formally equivalent to the S3 state and is close to the Mn cluster and has a similar lifetime to normal S3 (reviewed in Ref. [22]). The chemical identity of the free radical is unclear but experimental evidence has

been interpreted as indicating that it may be an oxidized histidine [40,41] or tyrosine [42]. Subsequent turnovers result in formation of the $P680^+Q_A^-$ radical pair which decays by charge recombination with a $t_{1/2}$ of approx. 150 μs [43]. Which of these events is directly associated with photodamage is unknown. Perhaps the most likely explanation is that $P680^+$ is able, with a low quantum yield, to oxidize nearby pigments (chlorophyll and/or carotenoids) leading to some irreversible damage in the reaction centre. This kind of mechanism has been suggested previously to explain photodamage in PSII under a range of conditions (e.g. Refs. [4,6,7,9,10,44]).

In conclusion, we consider that the absence of Ca^{2+}, and consequently the presence of the high potential form of Q_A, may result in electron transfer properties which allow dissipation of excess energy by a recombination reaction between Q_A^- and $P680^+$ which does not involve P680 triplet formation. Under prolonged illumination, photodamage still occurs and this may be due to the prolonged lifetime of $P680^+$ resulting in oxidation of near-by components. A more harmful situation occurs when Cl^- is depleted; electron donation from water is inhibited but the quinone remains in the low potential form. Illumination under these conditions is proposed to result in charge recombination forming P680 triplet which can interact with oxygen resulting in singlet oxygen formation and the associated non-specific damage. The peroxide reported to be formed by oxygen reduction in Cl^--depleted PSII may arise through a mechanism related to the redox form of Q_A, possibly via the reduction of singlet oxygen. Measurements of the recombination pathways occurring with high and low potential quinones are required to test these proposals.

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