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The role of cytochrome b_{559} and tyrosine$_D$ in protection against photoinhibition during in vivo photoactivation of Photosystem II

Ann Magnuson [a,*], Maria Rova [b], Fikret Mamedov [a], Per-Olof Fredriksson [b], Stenbjörn Styring [a]

[a] *Department of Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, Box 124, S-22100 Lund, Sweden*
[b] *Department of Chemistry, University of Karlstad, S-65188 Karlstad, Sweden*

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Abstract

In vivo photoactivation of Photosystem II was studied in the FUD39 mutant strain of the green alga *Chlamydomonas reinhardtii* which lacks the 23 kDa protein subunit involved in water oxidation. Dark grown cells, devoid of oxygen evolution, were illuminated at 0.8 μE m^{-2} s^{-1} light intensity which promotes optimal activation of oxygen evolution, or at 17 μE m^{-2} s^{-1}, where photoactivation compete with deleterious photodamage. The involvement of the two redox active cofactors tyrosine$_D$ and cytochrome b_{559} during the photoactivation process, was investigated by EPR spectroscopy. Tyrosine$_D$ on the D_2 reaction center protein functions as auxiliary electron donor to the primary donor P_{680}^+ during the first minutes of photoactivation at 0.8 μE m^{-2} s^{-1} (compare with Rova et al., Biochemistry, 37 (1998) 11039–11045.). Here we show that also cytochrome b_{559} was rapidly oxidized during the first 10 min of photoactivation with a similar rate to tyrosine$_D$. This implies that both cytochrome b_{559} and tyrosine$_D$ may function as auxiliary electron donors to P_{680}^+ and/or the oxidized tyrosine$^{\bullet}_Z$ on the D_1 protein, to avoid photoinhibition before successful photoactivation was accomplished. As the catalytic water-oxidation successively became activated, Tyrosine$_D$ remained oxidized while cytochrome b_{559} became rereduced to the equilibrium level that was observed prior to photoactivation. At 17 μE m^{-2} s^{-1} light intensity, where photoinhibition competes significantly with photoactivation, tyrosine$_D$ was very rapidly completely oxidized, after which the amount of oxidized tyrosine$_D$ decreased due to photoinhibition. In contrast, cytochrome b_{559} became reduced during the first 2 min of photoactivation at 17 μE m^{-2} s^{-1}. After this, it was reoxidized, returning to the equilibrium level within 10 min. Thus, during in vivo photoactivation in high-light cytochrome b_{559} serves two functions. Initially, it probably oxidizes the reduced primary acceptor pheophytin, thereby relieving the acceptor side of reductive pressure, and later on it serves as auxiliary electron donor, preventing donor-side photoinhibition.

Abbreviations: Chl, chlorophyll; cyt-b_{559}, cytochrome b_{559}; DCPIP, 2,6-dichlorophenolindophenol; DPC, 2,2′-diphenylcarbonic dihydrazide; E, einsteins; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; FUD 39, mutant strain of *Chlamydomonas reinhardtii* lacking the 23 kDa extrinsic protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P_{680}, the primary electron donor chlorophylls of Photosystem II; PS II, Photosystem II; Q_A and Q_B, the first and second quinone electron acceptors, respectively; SII_s, signal II_{slow}, the EPR signal from Tyr_D; Tris, tris(hydroxymethyl)aminomethane; Tyr_D and Tyr_Z, the redox active tyrosine residues on the D_2 and D_1 reaction center proteins, respectively; WOC, the water oxidizing complex of Photosystem II

* Corresponding author.

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1. Introduction

Photosystem II (PS II) is a large, multisubunit protein complex, located to the thylakoid membrane in plants, algae and cyanobacteria [1–3]. PS II catalyzes the light-driven reduction of plastoquinone by extracting electrons from water. The catalytic site for water oxidation consists of four manganese ions in the water oxidizing complex (WOC), which are located close to the lumenal side of PS II [4]. Electron transport from water to plastoquinone is carried out by the cofactors associated to the reaction center core proteins D_1 and D_2 [5]. Photo-oxidation of the primary electron donor chlorophylls P_{680}, leads to electron transfer to the primary acceptor pheophytin and, subsequently, to the quinones Q_A and Q_B on the acceptor side of PS II. On the donor side of PS II are two redox active tyrosines, known as Tyr_Z and Tyr_D in the D_1 and D_2 proteins, respectively [6]. The oxidized P_{680} is rereduced by Tyr_Z, which in turn is reduced from the manganese complex. Four consecutive oxidations of the manganese complex, lead to one turnover of the WOC and the oxidation of two water molecules to molecular oxygen [4,7,8]. To accomplish water oxidation, the oxidizing components in PS II reach and control very high redox potentials. These are potentially dangerous and may induce deleterious reactions, if electrons are not rapidly provided by the WOC [9,10]. Consequently, the integrity of the donor side of PS II is crucial.

During catalytic turnover, PS II is the target for so called photoinhibition, which leads to obstruction of the photochemistry, damage of the redox cofactors, and ultimately to degradation of the reaction center D_1 protein [9,11,12]. There are several mechanisms for photoinhibition, and one of them, so-called donor-side photoinhibition, is triggered by long-lived oxidizing radicals on the donor side of PS II [9,13,14]. Donor side-induced photoinhibition has been studied in PS II preparations depleted of a functional Mn cluster [10,15–17] depleted of chloride [14], or in PS II that retains the Mn cluster but lacks the extrinsic subunits that stabilize the water oxidizing complex [18,19].

Assembly of the entire PS II complex occurs during growth of the photosynthetic organism. De novo synthesis and insertion of the D_1 protein in PS II, also occurs continuously, to compensate for the degradation that follows on photoinhibition. When PS II is assembled, the last thing to be completed is the Mn cluster, which is incorporated in a light-dependent process known as photoactivation. Photoactivation is dependent on functional photochemistry in PS II and can only occur after the primary charge separation and secondary charge stabilizing reactions involving Tyr_Z and Q_A have become operational [20].

In the first two steps of photoactivation, the oxidized form of Tyr_Z provides the driving force for the oxidation of Mn^{2+} ions loosely bound to the water oxidizing site. The Mn^{3+} ions then bind strongly to the protein matrix [21–24]. The third and the fourth Mn ions bind to the site in a light independent way [24]. To function properly, the Mn cluster also has to bind the cofactors Ca^{2+} and Cl^-. These ions are concentrated in the WOC by three extrinsic subunits of 16, 23 and 33 kDa. The presence of both Ca^{2+} and Cl^- is necessary for photoactivation in vitro [25–27]. Correct Mn-ligation also includes a conformational change, which has to occur between the first and second photoligations of Mn^{2+}. The time necessary for this conformational rearrangement therefore puts a restriction on the light intensity required for optimal photoactivation [22–25,28].

Even if the main events in the process of photoactivation are fairly well understood, adequate knowledge is still lacking on several points. One central question is how PS II manages several charge separations without a functional water-splitting center that feeds in electrons, a situation where the risk of donor-side photoinhibition is high [9]. One protective mechanism is suggested to be that the redox potential of Q_A^- is decreased during in vitro photoactivation, from +110 mV in Mn-depleted PS II, to −80 mV when the PS II centers have become active in water splitting [29]. A higher redox potential of Q_A^- in inactive centers, results in a slower electron transfer rate from Q_A^- to Q_B, compared to active centers, thereby enhancing the probability for charge recombination between Q_A^- and the oxidizing $Tyr_Z^{\bullet}$ and $P_{680}{}^+$ radicals on the donor side. This will in turn decrease the life times of these species, and thus donor side-induced photoinhibition would be prevented.

In a recent study, performed in thylakoid membranes from dark-grown cells of *Chlamydomonas*

reinhardtii, we observed that the electron transfer on the acceptor side of PS II was slowed down [30]. Hence the rate for electron transfer out from PS II is slow before photoactivation. The study also revealed that the rate of the forward electron transfer from Q_A^- increases during in vivo photoactivation of water oxidation. Therefore, this change in electron transfer rate on the acceptor side, may be one method to protect PS II against photodamage early in the photoactivation process.

Another possibility would be if reduction of P_{680}^+ or $Tyr_Z^{\bullet}$ could be accomplished via auxiliary electron donors, when the manganese complex is unable to deliver electrons. In PS II devoid of the Mn cluster, oxidation of the slower donors Tyr_D and cyt-b_{559} has been observed [22,31,32]. Thus, albeit being poor electron donors to P_{680}^+ under normal conditions, Tyr_D and cyt-b_{559} may function as auxiliary reductants when the Mn cluster is absent, thereby protecting PS II against oxidative damage. Furthermore, cyclic electron transfer in PS II mediated by cyt-b_{559}, was proposed to act as a protection mechanism against the formation of oxidizing Chl radicals [33,34].

Here, we report EPR measurements of the oxidized forms of Tyr_D and cyt-b_{559}, during photoactivation of dark-grown cells from a mutant strain, FUD39, of *C. reinhardtii* and correlate the oxidation states of these cofactors to the assembly and function of the Mn complex. The FUD39 mutant is markedly slower in photoactivation, with a half-time for photoactivation of 10 min at optimal light conditions, compared to the wild type which photoactivates within 1–2 min [28]. The mutant is therefore very suitable for 'time resolved' investigations of processes that take place in the intermediate steps of photoactivation. We have compared photoactivation in the FUD39 cells at an optimal light intensity, when 60–70% of the centers can acquire water-splitting ability and the degree of photodamage is small, with photoactivation at a superoptimal light intensity, resulting in only about 30% active centers and extensive irreversible photoinhibition [28].

Our results show that the oxidation of Tyr_D takes place before the acquirement of water-splitting ability is complete, which corroborates its suggested role as an auxiliary electron donor. However, at photoinhibitory conditions, the requirement for Tyr_D as a rescuing electron donor is intensified, and the oxidation occurs faster. The behavior of cyt-b_{559} is more complex. At optimal photoactivating light intensities, cyt-b_{559} is oxidized with the same kinetics as Tyr_D. At higher light intensities however, cyt-b_{559} becomes reduced early in the photoactivation process, and then becomes oxidized later on. Thus, a picture emerges where cyt-b_{559} can act as both electron acceptor, and electron donor to prevent photoinhibition during in vivo photoactivation, thereby functioning as an internal redox buffer in PS II.

2. Materials and methods

2.1. Cell growth and photoactivation conditions

FUD39 mutant cells of *C. reinhardtii* were grown on Tris-acetate–phosphate medium in complete darkness at 23°C [35,36]. After 3–5 days of growth in the dark, the cells were in early exponential phase and no water-splitting activity was detected. Photoactivation was performed by illuminating the cells for various times in the light chamber as described previously [28,30], at well-defined light intensities of 0.8 and 17 μE m^{-2} s^{-1}, respectively. After the illumination, the cells were immediately placed on ice and harvested. The thylakoid membranes were prepared as described previously [30], by washing the cell culture in a buffer containing 25 mM Hepes–NaOH (pH 7.0) and 50 mM NaCl, breaking the cell walls by sonication, separating whole cells from membranes at $400\times g$ and collecting the thylakoids by centrifugation at $25\,000\times g$. 1 mM EDTA was added to the buffer after the first washing step, to avoid EPR signals from free Mn^{2+} ions. The thylakoids were suspended in a buffer with 25 mM Hepes–NaOH (pH 7.0), 50 mM NaCl, and 400 mM sucrose, and then frozen at −80°C before conducting EPR and electron transfer measurements. The cell harvesting and thylakoid preparation were performed in total darkness.

2.2. Electron transfer measurements

Electron transfer rates through PS II were measured spectroscopically as photoreduction of DCPIP at 590 nm, with or without the exogenous electron

donor DPC as described previously [28,30]. The rate of DCPIP reduction in the presence of the electron donor DPC, measures the total electron transport activity in all PS II centers. When DCPIP reduction is measured without addition of DPC, only those centers active in water-splitting are monitored. The quotient between the DCPIP reduction rate in the presence and absence of DPC therefore gives the percentage of PS II centers that are active in water oxidation [28,30].

2.3. EPR measurements

The thylakoid suspensions from the photoactivated cells without any additions were placed in EPR tubes in the dark, and frozen at 77 K, at a chlorophyll concentration of about 2 mg Chl/ml.

CW X-band EPR spectra were recorded at liquid helium temperatures with a Bruker ESP380 spectrometer, equipped with an Oxford Instruments helium flow cryostat and temperature controller. First the spectrum from the $Tyr_D^{\bullet}$ radical (Signal II_{slow}) was recorded in the membranes prepared from the photoactivated cells. Then, induction of the maximum amplitude of SII_s in the photoactivated samples was made by thawing the EPR samples and illuminating them for 5 min at 21°C with a 1000 W projector lamp. The samples were then dark adapted at room temperature for 5 min, to allow the signal from P_{700}^{+} (Photosystem I) to decay, and were frozen again at 77 K.

The maximal amplitude of $Tyr_D^{\bullet}$ represents one radical spin per PS II (there is one Tyr_D per D_2 protein). The amount of oxidized cyt-b_{559} in PS II from dark-grown cells was estimated from the amplitude of the g_z-tensor at $g \sim 3.0$, of the EPR-signal that is associated with the oxidized form of cyt-b_{559}. At $g \sim 3.0$ there is no interference with signals from the cyt-b/f_6 complex, for which the g_z-tensors have g-values of ~ 3.5 ([37], and references therein).

We compared the EPR signals in our samples, with those of highly oxygen evolving PS II membranes from spinach (so-called BBY particles), where after a short dark incubation, SII_s represents one $Tyr_D^{\bullet}$ radical spin per PS II center [6,38] while the amount of oxidized cyt-b_{559} reflects 0.4–0.5 spin per PS II center (assuming 1.0–1.2 cyt-b_{559} per PS II center) [31,38,39]. From a comparison of the EPR spectra from $Tyr_D^{\bullet}$ (induced to its maximum, see above) and cyt-b_{559}^{ox} (measured prior to any illumination) we could estimate cyt-b_{559}^{ox} to represent 0.9–1.0 spin per PS II center in thylakoid membranes from the dark-grown cells.

3. Results

3.1. Photoactivation and photoinhibition of electron transport

The mutant strain FUD39 from *C. reinhardtii*, which lacks the extrinsic 23 kDa protein subunit, was grown heterotrophically in the dark. Under these conditions, PS II is assembled but the PS II centers lack the Mn cluster. The cells were then photoactivated for various times at light intensities of 0.8 μE $m^{-2}\,s^{-1}$ or 17 μE $m^{-2}\,s^{-1}$, respectively, and harvested. In the thylakoid membranes, water oxidation activity was obtained in 60% of all the centers after photoactivation for 30 min at 0.8 μE $m^{-2}\,s^{-1}$, increasing at this light intensity with a half-time about of 10 min (Fig. 1B).

When photoactivation was conducted at the superoptimal light intensity 17 μE $m^{-2}\,s^{-1}$, photoinhibitory reactions competed with the photoactivation [28]. At this light intensity, water-splitting capacity was only achieved in 30–35% of the PS II centers (Fig. 2B). In addition, as the activation times were prolonged, the PS II centers were progressively photoinhibited, such that the yield of photoactivated centers decreased with increasing illumination times. However, PS II centers that had been photoactivated for less than 20 min, could be almost completely activated by decreasing the light intensity from 17 to 0.8 μE $m^{-2}\,s^{-1}$, indicating that the photoinhibition was reversible early during the photoactivation process (not shown, but see [28]). Longer illumination times resulted in successive irreversible photoinhibition similar to what was observed earlier [28]. In the present study we have utilized EPR spectroscopy to study secondary electron transport reactions involving secondary donors during photoactivation by following changes in the redox states of Tyr_D and cyt-b_{559}. Their participation, as electron donor and/or acceptors, in the in vivo photoactivation process has been investigated to gain more insight

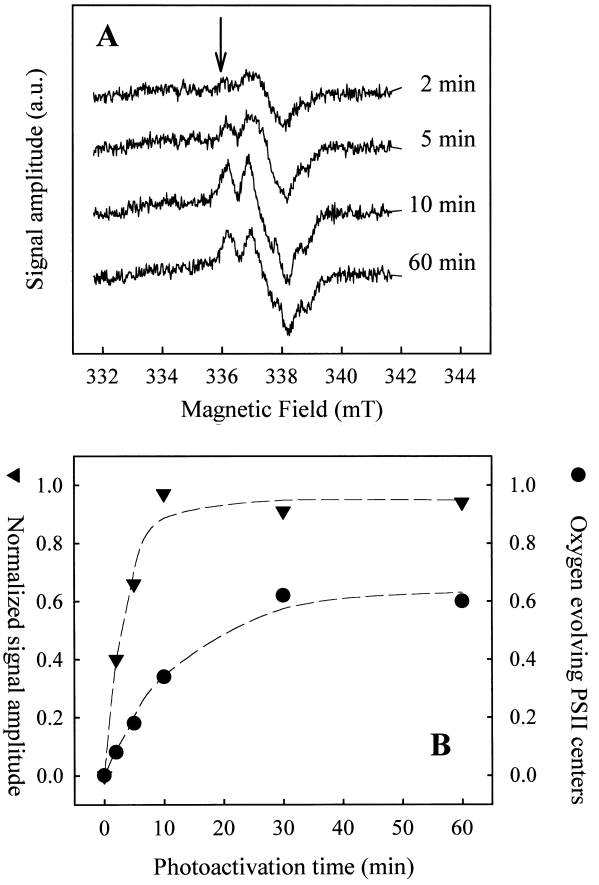

Fig. 1. Oxidation of Tyr_D during photoactivation at optimal light intensity (0.8 μE $m^{-2} s^{-1}$) of dark-grown cells of the FUD39 mutant of *C. reinhardtii*. (A) EPR spectra showing the induction of the $Tyr_D^{\bullet}$ radical (SII_s) with various times of photoactivating light. Tyr_D could be photo-oxidized to 100% in all samples in this series, as described in Section 2 (not shown, but see Fig. 3 in [30]). The EPR spectra are normalized with respect to sample concentrations. EPR settings. *T*, 15 K; microwave power 1.18 μW; microwave frequency 9.44 GHz; modulation amplitude 3.2 G. (B) Time-dependent induction of $Tyr_D^{\bullet}$ (triangles) compared to the time-dependent photoactivation of the oxygen evolving capacity (circles) at 0.8 μE $m^{-2} s^{-1}$. The half-time for the oxidation of Tyr_D was 4 min and for the activation of oxygen evolution 10 min. To avoid interference from other radical species in the $g = 2.00$ region, the signal amplitude of $Tyr_D^{\bullet}$ was estimated from the amplitude at the low field shoulder of the EPR signal indicated with an arrow in A.

into the complex process and to explain some phenomena during photoactivation.

3.2. Induction of SII_s from Tyr_D at optimal and superoptimal light intensities

The redox potential of Tyr_D has been estimated to +760 mV [40], and it can therefore be oxidized by either or both of P_{680}^{+} and $Tyr_Z^{\bullet}$. $Tyr_D^{\bullet}$ then remains oxidized for an indefinite time in the light, or for about 30 min in the dark depending on the redox state of the manganese cluster [40,41]. We did not

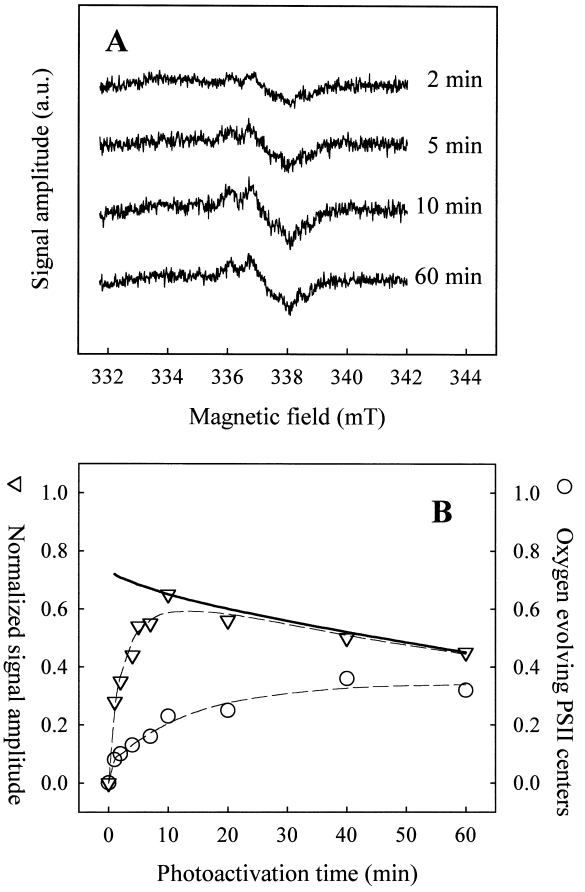

Fig. 2. Oxidation of Tyr_D during photoactivation at the superoptimal light intensity of 17 μE $m^{-2} s^{-1}$ of dark-grown cells of the FUD39 mutant of *C. reinhardtii*. (A) EPR spectra showing the induction and subsequent decrease of the $Tyr_D^{\bullet}$ radical with various times of photoactivating light. The EPR spectra are normalized with respect to sample concentrations, and EPR settings and can be compared to the spectra in Fig. 1A. Microwave power 1.82 μW, other EPR settings as in Fig. 1A. (B) Time-dependent induction and decrease of $Tyr_D^{\bullet}$ (triangles) compared to the time-dependent activation and subsequent inhibition of the oxygen evolving capacity (circles) at a light intensity of 17 μE $m^{-2} s^{-1}$. The full-drawn line represents the total fraction of centers that could obtain functional oxygen evolving capacity by transferring the cells, that had been illuminated at 17 μE $m^{-2} s^{-1}$ for the various times indicated, to the optimal light intensity of 0.8 μE $m^{-2} s^{-1}$ for another 30 min (compare Fig. 3 in [28]).

detect any SII_s, i.e., the EPR signal from $Tyr_D^{\bullet}$, in thylakoids from dark-grown cells, showing that Tyr_D was reduced in all PS II centers before photoactivation. We have earlier reported that SII_s increases in amplitude with increasing photoactivation times during early photoactivation [30], when the cells are activated under optimal light conditions, i.e., at 0.8 μE $m^{-2} s^{-1}$ m light intensity (Fig. 1A). The half-time for the oxidation of Tyr_D was 4 min at 0.8 μE $m^{-2} s^{-1}$ and SII_s reached its maximal amplitude after photoactivation for 10 min (Fig. 1B). Thus, at optimal conditions, the induction rate of SII_s was about twice the activation rate for the water-oxidizing complex. After reaching the maximum level (after 10 min), the amplitude of SII_s then remained constant as the membranes were illuminated for longer periods of time. By a control experiment, where the thylakoids were illuminated for 5 min at room temperature to induce the maximal amplitude of SII_s, we concluded that 100% SII_s, i.e., oxidation of Tyr_D in all centers, was accomplished after photoactivation for 10 min. The water-splitting activity was activated in 60% of the centers in the same cells (Fig. 1B, see also [28,30]).

When photoactivation was conducted at 17 μE $m^{-2} s^{-1}$, i.e., at super-optimal light conditions, Tyr_D was oxidized (Fig. 2B) at a slightly higher rate than in the membranes that were photoactivated at 0.8 μE $m^{-2} s^{-1}$ (Fig. 1B), with a half-time for the reaction of about 2–3 min. However, after 10 min of illumination at the superoptimal light intensity, the amount of oxidized Tyr_D was only 60–65% of the total (Fig. 2B). The rate for photoactivation of water oxidation at this superoptimal light intensity was very similar to that in membranes activated at optimal light intensity, i.e., photoactivation reached a maximum level after about 30 min, with a half-time of nearly 10 min. In these cells, the water-splitting capacity never reached more than 30–35% of the maximal level due to competing photoinhibitory reactions (Fig. 2B, and [28]). After 25 to 30 min of photoactivation, the water-splitting capacity did not increase any more (in some experiments it even decreased), an effect of the increasing irreversible photoinhibition following the prolonged illumination (Fig. 2B). Simultaneously with this, and in contrast to the situation in PS II centers activated at optimal light intensities, the amplitude of SII_s also started to decrease. After 60 min of illumination, only about 45% of SII_s remained oxidized (Fig. 2).

During the first 5 min of illumination at 17 μE $m^{-2} s^{-1}$, SII_s was induced to approximately the same extent as in the cells photoactivated at optimal light conditions. The rapid and substantial increase in SII_s during the first minutes, indicates that Tyr_D in these centers functioned well as electron donor to either P_{680}^{+} or $Tyr_Z^{\bullet}$ early in the process. However, with prolonged illumination times, the amplitude of the signal declined. In an earlier study, it was shown that the photoinhibition is reversible very early in the photoactivation process at 17 μE $m^{-2} s^{-1}$ and that full catalytic activity in the temporarily inhibited centers can be achieved by transferring the cells to lower light intensities (Fig. 1B, solid line, and [28]). By longer illumination times, the ability to regain full activity decreased, as irreversible damaging reactions increase. This was well reflected in the yield of oxidized Tyr_D, which was good during the first minutes of illumination and then started to decrease (Fig. 2B). These results indicate that the secondary electron transfer pathways were not inhibited early in the photoactivation. It also shows that the more severe effects of photoinhibition set in after about 10 min of superoptimal illumination.

3.3. Involvement of cytochrome b_{559}

Cyt-b_{559} is an integral component of the PS II reaction center [31,42]. The cytochrome shows a distribution of potential forms under different conditions; the high potential (HP) form, the intermediate potential (IP) and the low potential (LP) forms [31,43,44]. In PS II with an intact and fully functional water-splitting complex, cyt-b_{559} is prevalently in the reduced HP form. If the extrinsic 16 and 23 kDa protein subunits are removed or the water-oxidizing complex is inactivated completely, cyt-b_{559} is prevalently in the oxidized LP and/or IP form(s) [44]. Conversion of the LP form into the HP form has been observed during photoactivation [44–46]. Low yield photo-oxidation of the HP form has been observed in water-splitting PS II centers [47,48], and under photoinhibitory conditions [33,47]. In addition, reduction of the LP form has been reported [49] during photoinhibition.

A portion of oxidized cyt-b_{559} was observed in all

dark-grown, light-grown and photoactivated PS II samples (Fig. 3A). It was not reducible with hydroquinone but was possible to reduce to about 50% with 10 mM ascorbate (not shown). This indicates that the observed cyt-b_{559}^{ox} was in the IP, LP or very LP forms [31,44]. Photoactivation increased the amount of oxidized cyt-b_{559} (see below) as judged from its increased EPR signal (Fig. 3A). This fraction of cyt-b_{559} could be reduced quantitatively by the addition of 10 mM hydroquinone indicating that it was in the high potential form. The g-value and the width of the g_z EPR signal was identical in dark-grown and photoactivated samples at $g_z \cong 3.01$ (Figs. 3A,B) suggesting that this g-value reflects both the high and low potential forms of cyt-b_{559}.

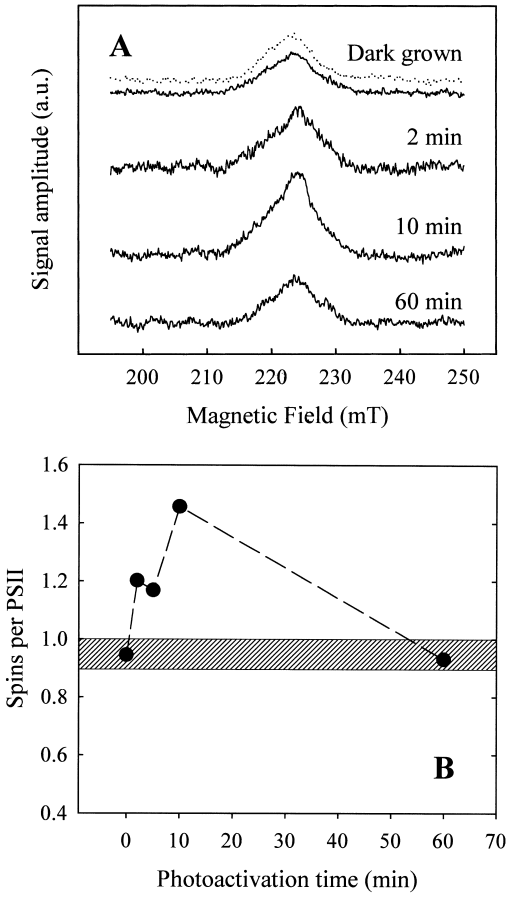

Fig. 3. Oxidation and reduction of cyt-b_{559} during photoactivation of dark-grown cells of the FUD39 mutant of *C. reinhardtii* at 0.8 μE $m^{-2} s^{-1}$ light intensity. (A) EPR spectra of the g_z-peak at $g \approx 3.0$ of oxidized cyt-b_{559} during photoactivation for various times. The full-drawn upper spectrum is from dark-grown cells. The dotted spectrum is from light-grown control cells. EPR conditions: T, 15 K; microwave power 11.2 mW; microwave frequency 9.44 GHz; modulation amplitude 15 G. All EPR spectra are normalized to the same sample concentration (different noise levels in the spectra are due to different concentration). (B) Time-dependent oxidation and reduction of cyt-b_{559} as deduced from the EPR spectra in A during photoactivation at 0.8 μE $m^{-2} s^{-1}$. The hatched zone indicates the variation in the amplitude of the oxidized cyt-b_{559} in samples from dark-grown and light-grown FUD39 mutant cells.

Very similar amplitudes, on a chlorophyll basis, of the $g \cong 3.0$ signal was observed in dark-grown samples and light-grown controls, indicating that dark- and light-grown cells of the mutant FUD39 contained approximately the same amount of oxidized cyt-b_{559} per PS II center (Fig. 3A, dotted line). By comparison with the EPR signals from $Tyr_D^{\bullet}$ and oxidized cyt-b_{559} in oxygen evolving PS II enriched membranes from spinach, the concentration of oxidized cyt-b_{559} from dark-grown cells was under the present conditions estimated to 0.9–1 oxidized cyt-b_{559} per PS II center (see Materials and Methods).

A rapid and substantial increase in the $g \cong 3.0$ signal was observed during the first minutes of photoactivation at 0.8 μE $m^{-2} s^{-1}$ (Figs. 3A,B). The observed fraction of oxidized cyt-b_{559} increased by 1.5 to 2 times compared to the level in the dark-grown membranes, and reached a maximum during the first 10 min of photoactivation, when the water-oxidizing complex was still largely unassembled (Fig. 1B). After longer photoactivation times, when the water oxidation activity had become fully evolved, the signal amplitude of cyt-b_{559} decreased again to a level close to that observed in dark-grown cells (Figs. 3A,B). The half-time for the early increase in the cyt-b_{559} EPR signal was approximately 5 min, coinciding with the half-time for maximum induction of SII_s at 0.8 μE $m^{-2} s^{-1}$ light intensity (Fig. 1). This indicates that cyt-b_{559}, similarly to Tyr_D in the same experiments, served as an auxiliary electron donor during early photoactivation at optimal light intensity. This occurs when a fully assembled and active manganese cluster is lacking while donation is not necessary once the manganese cluster is activated.

In cells that were photoactivated at 17 μE $m^{-2} s^{-1}$, a strikingly different redox behavior of cyt-b_{559} was observed. Instead of increasing by illumination time, as one might expect from the results of the illumination at 0.8 μE $m^{-2} s^{-1}$, the amplitude of the $g \cong 3.0$ signal decreased to less than half of that in the dark- or light-grown material during the first 2 min of il-

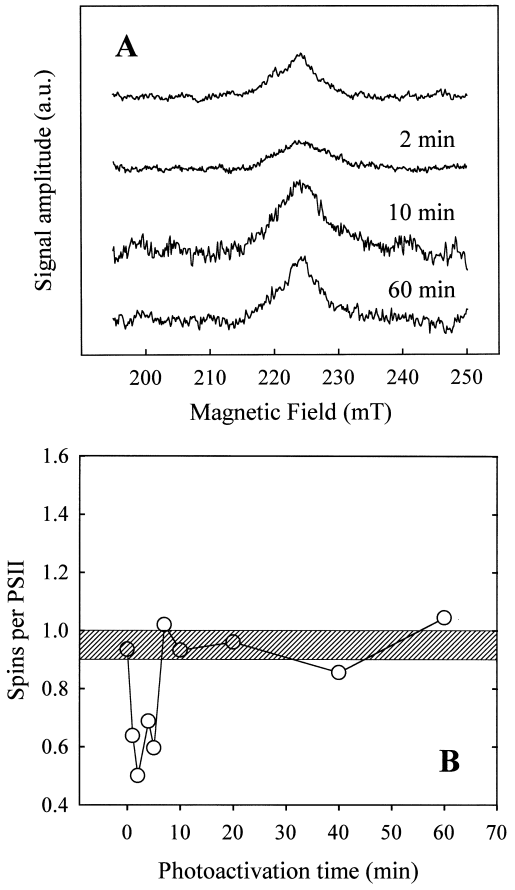

Fig. 4. Oxidation and reduction of cyt-b_{559} during photoactivation 17 μE $m^{-2} s^{-1}$ light intensity. (A) EPR spectra of the oxidized cyt-b_{559} during photoactivation for various times. The upper spectrum is from dark-grown cells. EPR conditions as in Fig. 3. (B) Time-dependent oxidation and reduction of cyt-b_{559} as deduced from the EPR spectra in A during photoactivation at 17 μE $m^{-2} s^{-1}$. The hatched zone indicates the variation in the amplitude of the oxidized cyt-b_{559} in samples from dark-grown and light-grown FUD39 mutant cells.

lumination (Figs. 4A,B). This reaction indicates that cyt-b_{559} rapidly became reduced in a large portion of the PS II centers. However, after the initial rapid reduction, by subsequently longer photoactivation times, cyt-b_{559} was reoxidized back to the same or a slightly higher level than in the dark-grown material. The half-time for this reoxidation was approximately 2 min, if calculated from the point where the amplitude started to increase after the initial reduction (Fig. 4B). Again, the oxidation rate of cyt-b_{559} was similar to that for Tyr_D, which in the same experiments became fully oxidized before the first 10 min, and with a half-time of 2–3 min. Hence, cyt-b_{559} again acted as electron donor in the absence of other, faster donors. It is likely that this function was masked during the first 2 min by a faster reductive reaction.

The initial reduction of cyt-b_{559}, observed when superoptimal light was applied, indicates that cyt-b_{559} served as an auxiliary electron acceptor during this treatment. However, since cyt-b_{559} obviously also became oxidized during a time interval after the first 2 min, up to 10 min of illumination, it apparently plays a flexible role, being accessible as both electron donor and acceptor under extreme external conditions.

4. Discussion

In our previous study of secondary electron transfer reactions during in vivo photoactivation, we could correlate the rate of SII_s induction (oxidation of Tyr_D), to events on the acceptor side of PS II [30]. During photoactivation of dark-grown cells at optimal light conditions (0.8 μE $m^{-2} s^{-1}$), we observed an increase of the Fv decay rates due to facilitated electron transfer from Q_A to Q_B, which clearly preceded the activation of the Mn cluster. The half-time for obtaining the maximum electron transfer rate from Q_A^- to Q_B (or Q_B^-) was 5 min, while the Mn cluster was assembled with a half-time of 10 min. Tyr_D was oxidized with a half-time of 5 min under the same conditions. A plausible interpretation for these data is that the increased forward electron transfer rate from Q_A^- to Q_B, and the resulting decrease in charge recombination between Q_A^- and $Tyr_Z^{\bullet}$, introduced a demand for Tyr_D to function as electron donor to $Tyr_Z^{\bullet}$, as long as the Mn-cluster still was largely unassembled.

Now we can also correlate the photo-oxidation of cyt-b_{559} during photoactivation at 0.8 μE $m^{-2} s^{-1}$ with the other events on the donor and acceptor sides of PS II and our conclusions are presented in Fig. 5. In Mn-depleted PS II centers, reduced cyt-b_{559} functions as electron donor to P_{680}^+, via a chlorophyll intermediate [31,33,47]. There have also been reports of low-yield oxidation of the HP form of cyt-b_{559} in PS II that are fully active in water-splitting [47,48]. It has consequently been suggested that cyt-b_{559} (in addition to Tyr_D) participates in slow

electron donation to the charge pairs P_{680}^{+}/Q_A^{-} and $Tyr_Z^{\bullet}/Q_A^{-}$ (which are in rapid equilibrium) in the absence of the Mn-cluster [32,33]. In the FUD39 mutant of *C. reinhardtii*, cyt-b_{559} is partly oxidized and partly reduced in the dark-grown algae (Fig. 3A). The EPR signal from the oxidized form of cyt-b_{559} increased during the first 10 min of in vivo photoactivation. The half-time for this oxidation was the same as for the induction of SII_s and involved the HP form of cyt-b_{559}. Apparently, the demand for electron donors to P_{680}^{+} and/or $Tyr_Z^{\bullet}$, which increased as forward electron transfer from Q_A^{-} became faster, also resulted in oxidation of cyt-b_{559}, until sufficient water-oxidation capacity had been reached (Fig. 5A). When the Mn cluster had become fully activated, there was no longer any need for auxiliary donors, and the oxidation of cyt-b_{559} ceased. Instead, the oxidized cyt-b_{559} became reduced (possibly via the Q_B^{-} or the PQ pool [47]) to the steady-state level that was observed in dark-grown and light-grown membranes. Thus, our results indicate that cyt-b_{559}, in the role as auxiliary electron donor, may be very important for safe and efficient photoactivation of PS II in vivo, also when this occurs under optimal conditions.

At a light intensity of 17 μE $m^{-2}\,s^{-1}$, which is higher than optimal very different reactions occur (Fig. 5B). SII_s was induced faster than at optimal light intensity (compare Figs. 1 and 2). However, it never reached its full amplitude. Instead, soon after reaching, what in this situation was, the maximum level (about 65% of Tyr_D oxidation after 10 min), SII_s started to decrease in amplitude. The decrease in SII_s was apparent already after about 15–20 min, whereas the water-splitting capacity reached its maximum at this time. Since this maximum in water-splitting activity only involved 30–40% of the PS II centers, the fast oxidation of Tyr_D in a large fraction of the centers suggests that at super optimal light Mn-clusters were under-represented as electron donors. Apparently, the demand for alternative electron donors became critical after only a few minutes of illumination at superoptimal light intensity and resulted in a fast exploitation of Tyr_D donation. However, the protective reactions were not efficient enough, and photoactivation competed with a signif-

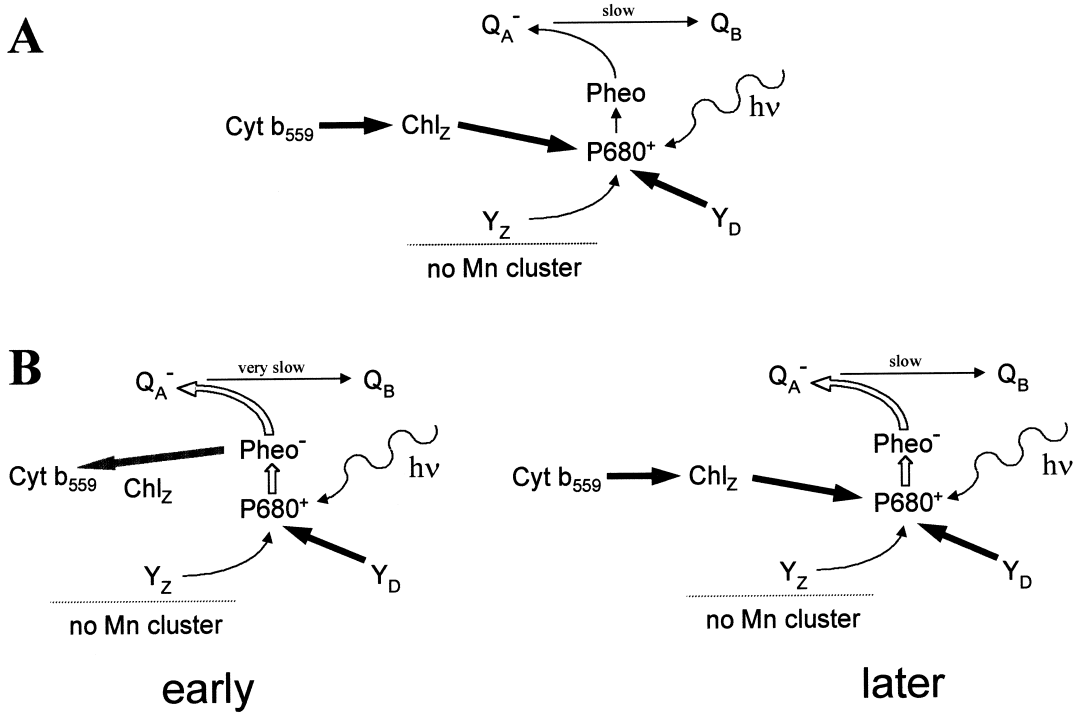

Fig. 5. Electron donation and abstraction pathways involving cyt-b_{559} and Tyr_D during early photoactivation of PS II prior to Mn-cluster assembly at 0.8 μE $m^{-2}\,s^{-1}$ (A) and 17 μE $m^{-2}\,s^{-1}$ (B) light intensities. The bold, black arrows indicate electron donation steps from Tyr_D or cyt-b_{559}, or reduction of cyt-b_{559} from reduced pheophytin.

icant and rapid photoinhibition which after some time became irreversible. This irreversible photoinhibition most probably involves damage and degradation of the D_1 protein, a process which has earlier been observed to occur on the same time-scale as Tyr_D destruction [50].

Under these conditions one would expect that also cyt-b_{559} would become photo-oxidized. Initially this seemingly did not occur however, but a fraction of cyt-b_{559} became rapidly reduced during the first 2 min of superoptimal illumination (Fig. 5B, left). This can probably be rationalized by reductive back reactions involving the reduced quinone acceptors and/or reduced pheophytin, which might form more frequently during illumination at 17 μE $m^{-2} s^{-1}$. In dark-grown algae, the forward electron transfer from Q_A^- is slow [30]. During photoactivation under superoptimal conditions, we recently observed a very early even more enhanced block of electron transfer from Q_A^- to Q_B. This was later changed to a normal forward electron transfer [51]. Therefore, early when PS II is subjected to high light intensity, there is an increased risk of over-reduction of the first quinone Q_A leading to a situation quite similar to acceptor side induced photoinhibition although the donor side is inefficient. The lethal double reduction of Q_A [52] can be slowed down by electron transfer from the primary acceptor pheophytin (Pheo) to a second component and it has been shown that cyt-b_{559} can protect PS II against acceptor side photoinhibition by accepting electrons from reduced Pheo [34,49,53]. It is therefore likely that the early reduction of cyt-b_{559} under high light intensity in our samples, can be explained by a similar process.

After the initial reduction of cyt-b_{559} at 17 μE $m^{-2} s^{-1}$, cyt-b_{559} again became oxidized (Fig. 5B, right). The oxidation occurred at the same rate as that for Tyr_D, but in contrast to what occurred at optimal light conditions, the oxidation level of cyt-b_{559} did not reach much higher than the equilibrium level in the dark-grown material. It is reasonable that this fast oxidation of cyt-b_{559} took place from the onset of illumination. However, initially it was masked by the reduction described above. If this scenario is true, the observed reduction and oxidation of cyt-b_{559} is the sum of two processes that occur in parallel and consequently involves a very large fraction of the PS II centers. One reason for this complex behavior could be that the cyt-b_{559} participated in a cyclic redox loop under these conditions. In such a loop the electrons would be transported from P_{680} to pheophytin, and then to cyt-b_{559}. However, albeit being slow, the electron transfer from Q_A^- to Q_B is not totally inhibited. In some PS II centers $Tyr_Z^{\bullet}$ can still form on the donor side. The oxidized Tyr_Z can have two fates that compete with each other: it can be reduced by Mn^{2+}, leading to photoactivation of the Mn cluster, although this is a low-probability event also under high light intensity, or $Tyr_Z^{\bullet}$ can be reduced by available donors, in particular cyt-b_{559} and Tyr_D. Thereby, the redox loop is closed, as cyt-b_{559} becomes reoxidized by P_{680}^+ and/or $Tyr_Z^{\bullet}$. This is supported by the observation that the rate of oxidation is the same for both cyt-b_{559} and Tyr_D, i.e., the oxidation rate for both these components seems to be governed by the same demand of auxiliary donors. A cyclic electron transport loop such as this might not be very efficient, but may hold irreversible photoinhibitory reactions at bay for at least a few minutes. Indeed, even at this high light intensity, a large fraction of PS II centers could be fully photoactivated if they were transferred from the inhibitory light to optimal light, as long as the cells were not illuminated for more than a few minutes [28].

5. Concluding remarks

Our results (Fig. 5) reveal an important aspect on the function of Tyr_D and cyt-b_{559} in PS II during photoactivation in vivo. The potential ability of both these cofactors to act as auxiliary electron donors to P_{680}^+ in the absence of the manganese cluster, seems to rescue the PS II reaction center against photodamage, by donating electrons during the time interval when electron transfer from Q_A to Q_B has become efficient, but before the water-splitting capacity is fully developed. In addition, the two components may protect PS II against both donor- and acceptor-side photoinhibition when the 'virgin' (developed in the dark) PS II centers are first subjected to high light intensities. The most intriguing role is played here by the cyt-b_{559} which can act both as electron donor and acceptor, for some time before irreversible photodestruction takes its toll.

As the photoactivation procedure in our study has been made in vivo, the results presented here shed new light on the physiological roles of cyt-b_{559} and Tyr_D. The integrity of the newly assembled PS II center, that lacks a functional Mn complex, is always jeopardized when it is hit by light the first time. If the light intensity is not too high, the internal protective mechanisms [30], i.e., slow forward electron transfer from Q_A^- and the auxiliary electron donation from Tyr_D and cyt-b_{559}, are enough to avoid photoinhibitory reactions and allow photoactivation. If the PS II center meets a high light intensity, however, it has to cope with two deleterious reactions at the same time: over-reduction of the acceptor side when forward electron transfer from Q_A^- is slow, and donor-side photoinhibition due to inefficient photoactivation at high light. In this case, the internally protective components Tyr_D and cyt-b_{559}, are oxidized and reduced in a complex manner. Under sustained high light illumination, these are not sufficient to fully protect PS II which eventually will become irreversibly photoinhibited.

Cyt-b_{559} is considered an enigmatic component in PS II [31] and whether it has one discrete function remains elusive. LP cyt-b_{559} functions as a slow acceptor to electrons from the acceptor side in PS II during photoinhibition [42,49,53] and HP cyt-b_{559} is a slow donor to P_{680}^+ (mediated by the nearby $chlorophyll_Z$) when the Mn complex is not capable to deliver electrons [31–34]. Together these, and similar observations in PS II reaction center preparations, have led to proposals where cyt-b_{559} is switched between the HP and the LP form to protect PS II against photoinhibition [31,34,48]. Our observations are in line with these hypothesis, which, until now, all are based on in vitro experiments. A newly assembled PS II without the Mn cluster might meet too high light. It is well protected and cyt-b_{559}, which is able to both give and take electrons, can fine tune the protection against photodamage even more (Fig. 5). We have observed about 1 oxidized LP (and IP) cyt-b_{559} in the dark-grown algae. Photoactivation led to oxidation of much more cyt-b_{559} (0.5–0.6 spins/PS II) which mainly were in the HP form. In is thus highly likely that PS II in dark-grown *C. reinhardtii* is equipped with two copies of cyt-b_{559}, one LP copy to prevent oxidative damage and one HP to prevent reductive damage due to over-reduction. We are presently investigating the detailed stoichiometry of the various potential forms of cyt-b_{559} during photoactivation.

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References

[1] B. Andersson, L.-G. Franzén, in: L. Ernster (Ed.), New Comprehensive Biochemistry: Molecular Mechanisms in Bioenergetics, vol. 23, Elsevier Science, Amsterdam, 1992, pp. 121–143.
[2] W.F.J. Vermaas, S. Styring, W.P. Schröder, B. Andersson, Photosynth. Res. 38 (1993) 249–263.
[3] B. Hankamer, J. Barber, E.J. Boekema, Annu. Rev. Plant. Phys. Mol. Biol. 48 (1997) 641–671.
[4] V.K. Yachandra, K. Sauer, M.P. Klein, Chem. Rev. 96 (1996) 2927–2950.
[5] B. Diner, G.T. Babcock, in: R. Ort, C.F. Yocum (Eds.), Oxygenic Photosynthesis: The Light Reactions, Kluwer Academic, Dordrecht, 1996, pp. 137–164.
[6] G.T. Babcock, B.A. Barry, R.J. Debus, C.W. Hoganson, M. Atamian, L. McIntosh, I. Sithole, C.F. Yocum, Biochemistry 28 (1989) 9557–9565.
[7] R.D. Britt, in: D.R. Ort, C.F. Yocum (Eds.), Oxygenic Photosynthesis: The Light Reactions, Kluwer Academic, Dordrecht, 1996, pp. 137–164.
[8] C. Tommos, G.T. Babcock, Acc. Chem. Res. 31 (1998) 18–25.
[9] C. Jegerschöld, S. Styring, in: N.R. Baker, J.R. Bowyer (Eds.), Photoinhibition of Photosynthesis, Bios Scientific, Oxford, UK, 1994, pp. 51–73.
[10] C. Jegerschöld, S. Styring, FEBS Lett. 280 (1991) 87–90.
[11] E.-M. Aro, I. Virgin, B. Andersson, Biochim. Biophys. Acta 1143 (1993) 113–134.
[12] O. Prasíl, N. Adir, I. Ohad, Biochim. Biophys. Acta 1102 (1992) 269–352.
[13] C. Jegerschöld, S. Styring, Biochemistry 35 (1996) 7794–7801.
[14] C. Jegerschöld, I. Virgin, S. Styring, Biochemistry 29 (1990) 6179–6186.
[15] D.J. Blubaugh, G.M. Cheniae, Biochemistry 29 (1990) 5109–5118.
[16] V.V. Klimov, M.A. Shafiev, S.I. Allakhverdiev, Photosynth. Res. 23 (1990) 59–65.

[17] D.J. Blubaugh, M. Atamian, G.T. Babcock, J.H. Golbeck, G.M. Cheniae, Biochemistry 30 (1991) 7586–7597.
[18] S.R. Mayes, K.M. Cook, S.J. Self, Z. Zhang, J. Barber, Biochim. Biophys. Acta 1060 (1991) 1–12.
[19] M. Rova, L.-G. Franzén, P.-O. Fredriksson, S. Styring, Photosynth. Res. 39 (1994) 75–83.
[20] K.-J. van Wijk, B. Andersson, S. Styring, Biochim. Biophys. Acta 1100 (1992) 207–215.
[21] N. Tamura, G.M. Cheniae, Biochim. Biophys. Acta 890 (1987) 179–194.
[22] A.-F. Miller, G.W. Brudvig, Biochemistry 29 (1990) 1385–1392.
[23] A.-F. Miller, G.W. Brudvig, Biochemistry 28 (1989) 8181–8190.
[24] R. Radmer, G.M. Cheniae, in: J. Barber (Ed.), Primary Processes of Photosynthesis, Elsevier/North Holland Biomedical, Amsterdam, 1977, pp. 303–348.
[25] M. Miyao, Y. Inoue, Biochemistry 30 (1991) 5379–5387.
[26] N. Tamura, Y. Inoue, G.M. Cheniae, Biochim. Biophys. Acta 976 (1989) 173–181.
[27] L. Zaltsman, G.M. Ananyev, E. Bruntrager, G.C. Dismukes, Biochemistry 36 (1997) 8914–8922.
[28] E.M. Rova, B. McEwen, P.-O. Fredriksson, S. Styring, J. Biol. Chem. 271 (1996) 28918–28924.
[29] G.N. Johnson, A.W. Rutherford, A. Krieger, Biochim. Biophys. Acta 1229 (1995) 202–207.
[30] M. Rova, F. Mamedov, A. Magnuson, P.-O. Fredriksson, S. Styring, Biochemistry 37 (1998) 11039–11045.
[31] D.H. Stewart, G.W. Brudvig, Biochim. Biophys. Acta 1367 (1998) 63–87.
[32] C.A. Buser, L.K. Thompson, B.A. Diner, G.W. Brudvig, Biochemistry 29 (1990) 8977–8985.
[33] L.K. Thompson, G.W. Brudvig, Biochemistry 27 (1988) 6653–6658.
[34] J. Barber, J. De Las Rivas, Proc. Natl. Acad. Sci. USA 90 (1993) 10942–10946.
[35] E.H. Harris, The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use, Academic Press, San Diego, 1989.
[36] D.S. Gorman, R.P. Levine, Proc. Natl. Acad. Sci. USA 54 (1965) 1665–1669.
[37] J. Bergström, L.-E. Andréasson, T. Vänngård, FEBS Lett. 164 (1983) 71–74.
[38] A.-F. Miller, G.W. Brudvig, Biochim. Biophys. Acta 1056 (1991) 1–18.
[39] C.A. Buser, B.A. Diner, G.W. Brudvig, Biochemistry 31 (1992) 11441–11448.
[40] I. Vass, S. Styring, Biochemistry 30 (1991) 830–839.
[41] S. Styring, A.W. Rutherford, Biochim. Biophys. Acta 933 (1988) 378–387.
[42] J. Whitmarsh, H.B. Pakrasi, in: R. Ort, C.F. Yocum (Eds.), Oxygenic Photosynthesis: The Light Reactions, Kluwer Academic, Dordrecht, 1996, pp. 249–264.
[43] W.A. Cramer, J. Whitmarsh, Annu. Rev. Plant Physiol. 28 (1977) 133.
[44] L.K. Thompson, A.-F. Miller, C.A. Buser, J.C. dePaula, G.W. Brudvig, Biochemistry 28 (1989) 8048–8056.
[45] N. Mizusawa, M. Ebina, T. Yamashita, Photosynth. Res. 45 (1995) 71–77.
[46] N. Mizusawa, M. Miyao, T. Yamashita, Biochim. Biophys. Acta 1318 (1997) 145–158.
[47] C.A. Buser, B. Diner, G.W. Brudvig, Biochemistry 31 (1992) 11449–11459.
[48] J.M. Ortega, M. Hervás, M.A. De la Rosa, M. Losada, Photosynth. Res. 46 (1995) 185–191.
[49] M. Poulson, G. Samson, J. Whitmarsh, Biochemistry 34 (1995) 10932–10938.
[50] C. Jegerschöld, S. Styring, Biochemistry 35 (1996) 7794–7801.
[51] M. Rova, F. Mamedov, A. Magnuson, P.-O. Fredriksson, S. Styring, in: G. Garab (Ed.), Proceedings of the XI International Congress on Photosynthesis, Kluwer Academic, Dordrecht, 1999 in press.
[52] S. Styring, I. Virgin, A. Ehrenberg, B. Andersson, Biochim. Biophys. Acta 1015 (1990) 269–278.
[53] L. Nedbal, G. Samsom, J. Whitmarsh, Proc. Natl. Acad. Sci. USA 89 (1992) 7929–7933.