Photoactivation and Photoinhibition Are Competing in a Mutant of *Chlamydomonas reinhardtii* Lacking the 23-kDa Extrinsic Subunit of Photosystem II*

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The process of photoactivation has been studied in dark grown cells of *Chlamydomonas reinhardtii*. A mutant, FUD 39, lacking the Cl\(^{-}\)-concentrating 23-kDa psbP protein of photosystem II was found to have a decreased capability to perform photoactivation. The yield of the process never reached wild type level, and contrary to the wild type, it was highly dependent on the intensity of the activating light, with a very narrow optimum around 1 \(\mu\)E m\(^{-2}\) s\(^{-1}\). The different behavior in the mutant can be explained by a requirement for a longer dark period, between the two photoacts, during the photoactivation. This is proposed to reflect the decreased Cl\(^{-}\) affinity in the mutant. Photoactivation in the mutant was also found to be very sensitive to competing photoinhibitory processes. The inhibition was located to the donor side of photosystem II and affected the photoactivation capability before electron transfer from Tyr\(_z\) was inhibited. We propose an extended model for photoactivation in which an intermediate that is sensitive to photoinhibition is formed if Cl\(^{-}\) is not functionally bound to the manganese cluster.

In oxygen-evolving photosynthetic organisms the oxidation of water is catalyzed by the manganese cluster of photosystem II (PS II)\(^1\) which is situated on the luminal side of the thylakoid membrane (for reviews on PS II and oxygen evolution, see Refs. 1–5), contains four manganese ions, and requires Cl\(^{-}\) and Ca\(^{2+}\) as cofactors to function properly (3, 6, 7). It was recently proposed that the functional entity involved in water oxidation includes not only the manganese cluster but also the nearby, redox active tyrosine residue on the D1 reaction center protein, Tyr\(_z\) (8–10). In addition, three extrinsic subunits on the luminal side of PS II, with molecular masses of 16–18, 23–24, and 33 kDa, respectively, are known to increase the efficiency of the water oxidizing complex (11–15). They act as concentrators of Cl\(^{-}\} and Ca\(^{2+}\}, and their presence enhances the binding of these essential ions. In the absence of the 23-kDa subunit the Cl\(^{-}\) affinity of PS II is decreased several orders of magnitude (7, 12, 16, 17). Furthermore the 33-kDa subunit stabilizes manganese binding, and in its absence two manganese ions are lost (18, 19).

In some organisms, including the green algae *Chlamydomonas reinhardtii*, PS II assembly occurs efficiently in the dark. During assembly, the complete PS II complex with all redox components, except for the manganese cluster, is formed, and PS II from dark grown *C. reinhardtii* is fully functional except for the water splitting capability. Manganese-deficient PS II can also be prepared by different washing procedures involving Tris washing and treatment with NH\(_4\)OH or hydroquinone. An active manganese cluster is formed in a manganese-deficient PS II center by a light-dependent process called photoactivation. This has been extensively studied both in vivo in dark grown cells or in vitro in purified preparations. Photoactivation is known to involve at least two successive photo reactions, in which manganese is photoligated to PS II, with a dark reaction in between (3, 20–23). The first photoreaction most likely involves oxidation of one Mn\(^{3+}\) to Mn\(^{4+}\) (24), and it is believed that the next photoreaction involves oxidation of a second Mn\(^{3+}\) to Mn\(^{4+}\) (23). During the dark reaction, the intermediate formed in the first photoreaction is converted to a second intermediate before the next photoreaction can occur. Both intermediates are unstable and decay back to a manganese-deficient state if the second photoreaction does not occur. To complete the manganese cluster, two more manganese ions bind to the cluster in a light-independent way after the second photoreaction.

Both Cl\(^{-}\} and Ca\(^{2+}\} are vital cofactors for oxygen evolution and also increase the efficiency of photoactivation. However, Ca\(^{2+}\} does not appear to be essential for the photoligation of Mn\(^{2+}\} but is required to achieve maximal activity of the water oxidizing complex (23, 25). The role of Cl\(^{-}\} during photoactivation is not well understood, and contradictory results about its importance exist. Tamura and Cheniae (23) reported a 2-fold increase in the photoactivation yield with the addition of Cl\(^{-}\}, whereas Miyao and Inoue (39) found a much stronger Cl\(^{-}\} dependence of the photoactivation process.

The electron transfer reactions in PS II without a functional manganese cluster are very sensitive to so-called donor side induced photoinhibition which involves light-triggered inhibition of partial reactions in PS II which later are followed by degradation of the D1 reaction center protein. Donor side photoinhibition is due to the accumulation of highly oxidizing components like P\(_{680}\}) and Tyr\(_z\} (26–29). The first partial reaction that is photoinhibited seems to be the ability to photoligiate Mn\(^{3+}\} during photoactivation (30), while electron transfer involving Tyr\(_z\) and P\(_{680}\} is inactivated in later stages.
(29, 30). The light sensitivity of the photoactivation process suggests that photoinhibition and photoactivation might be competing processes during assembly of PS II where the last step involves photoactivation of the manganese cluster in an otherwise fully functional PS II.

To gain further insight into the process of photoactivation, we have studied this process in vivo in dark grown cells of C. reinhardtii, the wild type, and the mutant FUD 39. We have previously shown that this mutant, lacking the 23-kDa extrinsic subunit of PS II, contained wild type levels of PS II (17). However, it showed reduced oxygen evolution that was activated only at quite high concentrations of Cl\(^{-}\). In our study, about 60% of the centers were active in water oxidation at a Cl\(^{-}\) concentration saturating the oxygen evolution, in contrast to the wild type where 100% of the centers were active. The remaining 40% of PS II centers were active in electron transport but could not use water as substrate. Furthermore, the water splitting capacity in thylakoid membranes of FUD 39 was also very susceptible to donor side photoinhibition and Cl\(^{-}\) protected against photoinhibition with a similar dissociation constant as it activated the oxygen evolution.

The decreased affinity for Cl\(^{-}\) in the oxygen-evolving center (OEC) of this mutant might also influence a potential Cl\(^{-}\)-dependence of the photoactivation process. If so, the lower oxygen-evolving capacity in FUD 39, compared with the wild type, could reflect a slower and less efficient photoactivation process in the mutant. That might also induce severe competition from donor side photoinhibition during photoactivation.

We have tested this hypothesis by studying photoactivation of FUD 39 at different light intensities. Our results show that photoactivation is less efficient and much more dependent on the intensity of the activating light in the mutant than in the wild type. This suggests an important role of Cl\(^{-}\) in the process of photoactivation. Interestingly, the competition between photoactivation and donor side photoinhibition is more pronounced in the mutant which leads to a situation where inhibition and activation of the manganese cluster occur in parallel. This is in accordance with the ability to perform photoactivation being the first step that is inactivated in donor side photoinhibition.

A hypothetical scheme explaining the coupling between photoactivation and photoinhibition and the involvement of Cl\(^{-}\) is presented.

**MATERIALS AND METHODS**

**Growth and Photoactivation Conditions**—C. reinhardtii, wild type, and FUD 39 were grown on Tris acetate/phosphate medium (31) in complete darkness at 23 °C, for 3–7 days. When the cells were in the early exponential growth phase, they were transferred to another growth chamber and exposed to illumination of various intensities for different periods of time to achieve photoactivation and/or photoinhibition.

The light was applied from light tubes (Osram L36W/41), and the intensity was regulated with neutral density filters. The spectral composition of the light was essentially the same at the different intensities. Light intensity was measured with a PAR sensor (SED/SHEL033/ PAR/W) adapted to a photometer (IL1400A radiometer (International Light, U. S. A.)). The temperature of the light chamber was held at 23 °C. The cells were transferred to ice immediately after the illumination period and harvested within 15 min after the end of photoactivation.

**Thylakoid Membrane Preparation**—Thylakoid membranes were prepared immediately after the photoactivation treatment as described earlier (17). All preparation steps were undertaken in total darkness or in very weak green light at 0–4 °C. No photoactivation occurred during the preparation of the thylakoid membranes, and control wild type or mutant samples that had not been preilluminated showed no water splitting activity. The thylakoid membranes were kept on ice in darkness until the electron transfer measurements that were concluded within a few hours after the preparation. Storage on ice during this time did not result in modification of any of the measured parameters.

**Electron Transfer Measurements**—The electron transfer through PS II was measured as photooxidation of 2,6-dichlorophenolindophenol (DCPIP) at 590 nm, with or without the exogenous donor 2,2'-diphe-
in good agreement with our analysis of the electron transfer measurements (Table I).

Light Intensity Is Critical for the Efficiency of the Photoactivation Process in FUD 39—The two time courses in Fig. 1 corresponding to an intensity of the activating light of 1 and 17 μE m⁻² s⁻¹, respectively, were quite similar for the wild type. In contrast, in the mutant the photoactivation yield varied much with the light intensity. At 17 μE m⁻² s⁻¹ only 50% of the maximal yield at 1 μE m⁻² s⁻¹ was reached. To explore the significance of the intensity of the activating light for the efficiency of photoactivation, we performed the experiment in Fig. 2A. It shows the time course for photoactivation of FUD 39 at light intensities between 0.2 and 60 μE m⁻² s⁻¹. Interestingly, there were several different and quite dramatic effects of varying the light intensity.

At low intensity there was a threshold for activation between 0.2 and 0.4 μE m⁻² s⁻¹. Below that, no activation of the oxygen-evolving complex occurred for at least 150 min (not shown). Above this threshold, the final yield of water splitting activity increased with light intensity until around 1 μE m⁻² s⁻¹ where 70% of the maximal DPC-mediated electron transfer capacity was reached. Half-time for activation was between 6 and 10 min at these light intensities, and the maximal activity was reached after 35–45 min of illumination. However, at higher light intensities the maximal level of water oxidation decreased again. With an increase of light intensity from 1 to 2 μE m⁻² s⁻¹, the yield is reduced from 70 to 45%. Activation with 60 μE m⁻² s⁻¹ results in only 15% activity of the water oxidizing center. At higher intensities there was also a gradual change toward faster photoactivation kinetics being most pronounced in the trace with 60 μE m⁻² s⁻¹ where the half-time for activation is 2 min and the time to reach the maximal activity is about 18 min, indicating disturbance from a competing inhibitory process (see below).

In Fig. 2B, the maximal activity reached after photoactivation is plotted against the intensity of the activating light. It clearly shows the differences between the two cell types. FUD 39 requires a higher light intensity to become photoactivated. While the wild type was fully activated at 0.2 μE m⁻² s⁻¹, no activation occurred in the mutant. The interval of light intensities capable of inducing maximal activity was very narrow in FUD 39. This is in sharp contrast to the broad optimum shown for the wild type. The differences were especially pronounced at higher light intensities where the process in the wild type was much more efficient. At 300 μE m⁻² s⁻¹, the wild type was still fully activated. In contrast, at 60 μE m⁻² s⁻¹ only 15% activation was reached in the mutant. It should be stressed that the reduction rate of DCPIP in the presence of DPC was constant (on chlorophyll basis) during the whole period of illumination regardless of the illumination regime. This means that the function of PS II reaction centers, except for the manganese cluster, was not affected by irreversible photoinhibition in these samples.
The cells were also stable up to 10 min when exposed to illumination at high light intensities, described above. The other was a decrease in the water oxidizing activity with prolonged illumination. The latter inhibition could be an effect of irreversible photoinhibition of active cells as shown for light grown cells illuminated by 60 \( \mu E \) \( m^{-2} s^{-1} \). However, illumination at 17 \( \mu E \) \( m^{-2} s^{-1} \) should not produce such an inhibition.

The question then arises whether the apparent inhibition seen at high activating light intensities involves irreversible damage of the water oxidizing complex or whether the cells could be fully photoactivated when brought back to optimal conditions for photoactivation. This was tested in the experiment presented in Fig. 3. Dark grown cells of FUD 39 were illuminated for various times at 17 \( \mu E \) \( m^{-2} s^{-1} \). They were thereafter transferred to 0.8 \( \mu E \) \( m^{-2} s^{-1} \) for 40 min (optimal conditions for photoactivation Fig. 2A). In such an experiment irreversible photoinhibition should be detected. The results show that after short (<10 min) illumination times at the high intensity, a second illumination at an optimal intensity resulted in complete photoactivation. Thus, no irreversible photoinhibitory damage of the photoactivating capacity had occurred. However, when the samples were exposed to high light for more than 10 min and then transferred to the low light intensity, less than maximal levels of photoactivation were reached. It is also seen that the final level of photoactivation achieved after the low light treatment decreased in correlation with the preillumination time at high light. Moreover, the reduction of the photoactivating capacity occurred in parallel to the decrease seen in the final level of activation (Fig. 3). Our interpretation of these results is that the decrease in the yield of photoactivation represents irreversible photoinhibition of the water oxidation site at high light intensities. The inhibition was specific to the capacity to form a functional water oxidizing complex and did not involve DPC-mediated electron transfer involving Tyr \(_{A}\). Furthermore, it only occurred in the FUD 39 mutant and not in the wild type at the light intensities studied.

**DISCUSSION**

Photoactivation involves a complex series of events. The first light reaction gives rise to an unstable intermediate that is converted to a second intermediate during a necessary dark period (3, 22). These steps are kinetically quite well described, but their chemical nature is not well understood. In this paper we report our results in a mutant of *C. reinhardtii*, FUD 39, lacking the 23-kDa extrinsic subunit of PS II. One important...
The effect of this mutation is that the mutant needs high concentrations of chloride to perform efficient water oxidation (17). At low light intensities the mutant is less efficiently photoactivated than the wild type. Furthermore, the mutant seems to never reach maximal possible levels of water splitting, and there are processes competing with photoactivation in which PS II centers become photoinhibited, both reversibly and irreversibly. Interestingly, efficient photoactivation in the mutant can only be accomplished over a very narrow light interval.

A light intensity of 0.2 μE m⁻² s⁻¹ is sufficient to fully photoactivate the wild type, while no activation at all is observed in FUD 39 (Fig. 2B). We see two possible reasons for this difference: 1) either one or more of the photoactivation intermediates is/are more unstable in the mutant compared with the wild type or 2) the intermediates are equally stable in both cell types, but FUD 39 requires a longer dark period, between the two photoacts, for efficient photoactivation. This would also lead to enhanced decay of the first intermediate. From the experiments performed at low light intensities alone, it is difficult to distinguish between these two hypotheses. However, from the experiments performed at superoptimal light (≥2 μE m⁻² s⁻¹, Figs. 2, A and B, and 3) some further conclusions can be drawn. Above 1 μE m⁻² s⁻¹ the maximal yield of photoactivation decreased abruptly. Despite this, the reduction rate of DCPIP in the presence of DPC was constant throughout the experiment indicating that most of the photochemistry in PS II, including Tyr, and the quinone acceptors, remained functional. Furthermore, the capacity to perform photoactivation was only reversibly inhibited during the first 10 min of illumination even at high light intensities (Figs. 3, 17 μE m⁻² s⁻¹). These results suggest that excessive illumination initially gives rise to nonproductive but not damaged species. Further illumination (>10 min), however, resulted in irreversible inhibition of the photoactivating capacity (Fig. 3) in accordance with reports that the ability to perform photoactivation is photoinhibited prior to other electron transfer reactions in PS II (28, 30, 38).

If the difference in the photoactivation mechanism between FUD 39 and the wild type was only the stability of the photoactivation intermediates (alternative 1), this would not result in decreased efficiency of photoactivation at superoptimal light intensities. Instead, when photons arrive faster, the photoactivation yield would continue to increase until the maximal level was reached or the photoactivating ability was photoinhibited. This is in conflict with the experimental results, since superoptimal light intensities gave lower yields of photoactivated centers compared with optimal light also in the first 10 min of illumination when no irreversible photoinhibition had occurred (Fig. 3).

In contrast, if the mutant needs a longer dark period for efficient photoactivation (alternative 2), our results could be qualitatively explained. At high light intensities the second photon would arrive prior to correct formation of the second intermediate. This is a potentially dangerous situation that could lead to photoinhibition since it might involve over-excitation of an incorrectly assembled manganese cluster. If this hypothesis is correct, the question arises why does the mutant need a longer dark period than the wild type and can we learn something about photoactivation in general from our experiments? The most obvious change of the mutation is that Cl⁻ is bound less efficiently to PS II. Despite this, correct binding of Cl⁻ is necessary for assembly of a functional manganese cluster. It is thus an attractive hypothesis that a longer dark period in the mutant would reflect the inefficient Cl⁻ binding. In Scheme 1, we propose an extended hypothetical model of the photoactivation mechanism that accounts for our results. The model involves a step in which Cl⁻ is bound to the partially assembled manganese cluster before the next photoreaction can occur. After the first photoreaction (I) the unstable intermediate B is formed. This intermediate is thought (24) to contain one Mn⁺⁺ ligated to PS II. It is generally believed that it has to be rearranged in darkness to the second unstable intermediate prior to the next photoreaction (IV). The reactions during the dark period are not known, but they probably involves a conformational change of the protein complex (II) that for example could open up the binding site of the next Mn⁺⁺. It is also possible that the dark period involves binding of Cl⁻ and Ca²⁺ to the partially assembled manganese cluster. In our study we cannot assess Ca²⁺ binding. However, in the FUD 39 mutant, the Cl⁻ binding affinity is severely lowered. We hypothesize that an unstable intermediate, Cinactive, is formed after the conformational change. This intermediate has to bind Cl⁻ (III) before a more stable intermediate, Cactive, is formed. This new intermediate is then able to proceed through the second photoreaction to form the productive intermediate D which thereafter is transformed to an active manganese cluster.

With Scheme 1 we can explain the difference between FUD 39 and the wild type both at low and high light intensities. In wild type, the Cl⁻ concentration at the OEC is high enough for a rapid conversion of Cinactive to Cactive. In the mutant, however, Cl⁻ binding is slow with a higher proportion of decay of Cinactive as a result. Therefore, the quantum yield for the whole process will be lower in the mutant as compared with the wild type accounting for the difference in efficiency between the two cell types at low light intensities. It is also interesting to note that a stabilizing effect by Cl⁻ on the second intermediate of photoactivation has been described (39) which is in accordance with Cinactive being more unstable than Cactive.

At higher than optimal light intensities, it is highly likely that a significant fraction of the PS II centers in the mutant

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**Scheme 1**

- **A**
  - Qₐ, Qₘ
  - hv
  - Tyr
  - Mn⁺⁺

- **B**
  - Qₐ, Qₘ
  - hv
  - Tyr
  - Mn⁺⁺

- **C**
  - Qₐ, Qₘ
  - hv
  - Tyr
  - Mn⁺⁺

- **D**
  - Qₐ, Qₘ
  - hv
  - Tyr
  - Mn⁺⁺

- **E**
  - 2Mn⁺⁺
  - hv

- **F**
  - Nonproductive state

- **G**
  - Active oxygen evolving PS II

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**Scheme 1 Description**

- **I**
  - Qₐ, Qₘ
  - hv
  - Tyr
  - Mn⁺⁺

- **II**
  - Qₐ, Qₘ
  - hv
  - Tyr
  - Mn⁺⁺

- **III**
  - Qₐ, Qₘ
  - hv
  - Tyr
  - Mn⁺⁺

- **IV**
  - Qₐ, Qₘ
  - hv
  - Tyr
  - Mn⁺⁺

- **V**
  - 2Mn⁺⁺
  - hv

- **VI**
  - hv

- **VII**
  - hv

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**Scheme 1 Notes**

- **Nonproductive state**
  - Mn⁺⁺
  - Mn⁺⁺

- **Active oxygen evolving PS II**
  - Cl⁻
receives a second photon, and thereby photoligates a second Mn$^{2+}$, prior to correct chloride binding (VI). We hypothesize that this will produce a nonproductive state, C*, with two possible fates. (i) Further illumination with high light intensities will irreversibly photoinhibit the PS II center (F). (ii) Lowering the light intensity would allow an increased proportion of C* to decay back to C$_\text{inactive}$ before the PS II has become irreversibly photodamaged. This behavior of C* would explain both the reversible and the irreversible photoinhibitory phases (Fig. 3). It should also be noted that when the complex has once reached the productive C$_\text{active}$ intermediate, photoactivation proceeds to the active manganese cluster by binding two further manganese ions (V).

Recently, it was reported that the redox potential of Q$_A$/QA$^-$ is increased by about 150 mV after Ca$^{2+}$ depletion or in the absence of the manganese cluster (40, 41). The increased potential of Q$_A$/QA$^-$ slows down the electron transfer between Q$_A$ and QA (42). This lowers the electron withdrawing capacity of the acceptor complex in PS II and was proposed to protect PS II against donor side photoinhibition in the absence of the manganese cluster (40). It was also observed that rebinding of manganese to a functional manganese cluster reversed the redox potential change of Q$_A$/QA$^-$ but it was not clear to what extent the manganese cluster had to be assembled (partially or completely) to induce the potential shift of Q$_A$$. Therefore, in Scheme 1, at least intermediate A should be protected against photoinhibition by this mechanism.

The manganese site-induced redox change of QA$^-$ is most likely mediated by conformational changes in the protein accompanying manganese and/or Ca$^{2+}$ binding. Therefore, it is possible that manganese ions in partially or incorrectly assembled manganese-clusters might also invoke the redox potential change (Scheme 1, pathway from B to F via C*). If so, the erroneous assembly of the manganese cluster in the mutant, due to too fast arrival of photons, could open the QA to QA electron transfer. This would eventually promote efficient electron withdrawal in PS II although the manganese cluster is incompetent. This situation is prone to donor side photoinhibition and would result first in inhibition of the manganese cluster and later in inhibition of Tyr$_F$. In this respect it is interesting that Cl$^-$ depletion does not result in a potential rise of QA$^-$.$^2$ This means that, in FUD 39, PS II centers formed during photoactivation, which lack Cl$^-$, would not be protected against photoinhibition. This is seemingly in accordance with our results that FUD 39 is more liable to photoinhibition during photoactivation than the wild type.

Although Scheme 1 might explain our observations, several aspects need further experimental work. It is for example known that PS II (in particular the manganese cluster) is less stable in the absence of the extrinsic subunits and Cl$^-$ also in the dark (18, 43), which might be of relevance for the photoactivation process in the FUD 39 mutant. However, the destabilization of PS II studied here is light-dependent and thus most likely reflects competition between photoactivation and photoinhibition, which is a different phenomenon.

Our results are relevant for several aspects of PS II research. We have described a mutant system where the balance between photoactivation and photoinhibition is clearly exposed which facilitates its studies. The acceptor side induced photoinhibition is well known and has been observed at high light intensities in vivo. The importance of donor side induced photoinhibition in vivo is less clear since the manganese cluster normally efficiently provides electrons to the potentially damaging radicals P$_{680}^0$ and Tyr$_{z^*}$. However, in PS II where the manganese cluster is malfunctioning or absent it might become important. Our hypothesis was that the early stages of photoactivation could involve such photosensitive states since the manganese cluster is absent. Our results in the FUD 39 mutant proves that this can indeed be true and that protective mechanisms cannot fully prevent photoinhibition. However, in the wild type this seems not to be a very important mechanism due to the higher efficiency of the photoactivation. Furthermore, our observation that photoactivation might be active only over a very narrow range of light intensities is of potential relevance in photosynthesis research when mutants in the manganese cluster or involving manganese binding are studied. It is likely that, in some mutants, photoinhibition might efficiently compete with photoactivation under normal conditions leading to erroneous conclusions on the effects of the mutation. In addition our results suggest that Cl$^-$ binding might be involved in the early stages of photoactivation and that excess light in the absence of Cl$^-$ might lead to the accumulation of incorrectly assembled manganese clusters. We are presently testing this hypothesis by studying photoactivation intermediates in FUD 39 by EPR spectroscopy.

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