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Light-Dependent Degradation of the D1 Protein in Photosystem II Is Accelerated after Inhibition of the Water Splitting Reaction†

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ABSTRACT: Strong illumination of oxygen-evolving organisms inhibits the electron transport through photosystem II (photoinhibition). In addition the illumination leads to a rapid turnover of the D1 protein in the reaction center of photosystem II. In this study the light-dependent degradation of the D1 reaction center protein and the light-dependent inhibition of electron-transport reactions have been studied in thylakoid membranes in which the oxygen evolution has been reversibly inhibited by Cl^- depletion. The results show that Cl^--depleted thylakoid membranes are very vulnerable to damage induced by illumination. Both the D1 protein and the inhibition of the oxygen evolution are 15–20 times more sensitive to illumination than in control thylakoid membranes. The presence, during the illumination, of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) prevented both the light-dependent degradation of the D1 protein and the inhibition of the electron transport. The protection exerted by DCMU is seen only in Cl^--depleted thylakoid membranes. These observations lead to the proposal that continuous illumination of Cl^--depleted thylakoid membranes generates anomalously long-lived, highly oxidizing radicals on the oxidizing side of photosystem II, which are responsible for the light-induced protein damage and inhibition. The presence of DCMU during the illumination prevents the formation of these radicals, which explains the protective effects of the herbicide. It is also observed that in Cl^--depleted thylakoid membranes, oxygen evolution (measured after the readdition of Cl^-) is inhibited before electron transfer from diphenylcarbazide to dichlorophenolindophenol. The latter activity is dependent on functional electron transfer in photosystem II between the electron donor tyrosine Z and the first quinone acceptor, Q_A. The kinetics for the inhibition of the electron transfer from diphenylcarbazide to dichlorophenolindophenol were approximately similar to the kinetics for the degradation of the D1 protein. Together these results indicate that the light-dependent degradation of the D1 protein is triggered by the accumulation of P_{680}^+ and/or tyrosine Z^+, both of which are highly oxidizing. It is proposed that similar reactions also trigger the degradation of the D1 protein in vivo and possible mechanisms for this are discussed.

Photosystem II (PSII)[1] is a large, multisubunit enzyme (Andersson & Åkerlund, 1987) that catalyzes the light-driven reduction of plastoquinone with electrons derived from water (Andréasson & Vänngård, 1988; Rutherford, 1989). The reaction center in PSII is composed of two hydrophobic proteins, D1 and D2, which are homologous of each other and of the L and M subunits in the photosynthetic reaction center from purple bacteria. The heterodimer of the L and M sub-

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[1] Abbreviations: DPC, 2,2′-diphenylcarbonic dihydrazide; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P_{680}, primary electron donor chlorophyll(s) of PSII; Pheo, pheophytin that functions as the intermediate electron acceptor to P_{680}; PSI, photosystem I; PSII, photosystem II; Q_A, first quinone acceptor in PSII; Q_B, second quinone acceptor in PSII; S_0–S_4, charge storage states of the oxygen-evolving complex; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Tyr_Z, tyrosine 161 on the D1 protein—electron carrier between P_{680} and the Mn cluster; Tyr_D, tyrosine 160 on the D2 protein—accessory electron donor in PSII.

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units binds the redox components necessary for the primary photochemistry (Deisenhofer et al., 1985). From sequence analogies (Michel et al., 1986; Trebst, 1986; Michel & Deisenhofer, 1988) and from functional similarities between the two systems (Rutherford, 1986) it is today established that the D1/D2 heterodimer in PSII (Nanba & Satoh, 1987) is built similar to the bacterial reaction center with respect to the protein structure and to the binding and function of several of the components that are involved in the photosynthetic electron transport through PSII (Andréasson & Vänngård, 1988; Rutherford, 1989).

The turnover of the D1 protein is much faster than of any other chloroplast protein [for a recent review, see Mattoo et al. (1989)]. This is due to light-dependent degradation of the protein and occurs in vivo under normal light conditions (Kyle et al., 1984; Kyle, 1985; Kirilovsky et al., 1988). The degradation is accelerated by strong illumination (Kyle, 1985) and has therefore been correlated with photoinhibition, which occurs at high light intensities and results in inhibition of the photosynthetic electron transfer through PSII due to limitations on the reducing side (Powles, 1984; Vass et al., 1988, Styring et al., 1990). In addition, the D2 protein is degraded faster than other chloroplast proteins (with the exception of the D1 protein). The irreversible degradation of the PSII reaction center proteins is accompanied by the de novo synthesis of the D1 protein and its incorporation into newly assembled PSII units (Kyle, 1985; Mattoo et al., 1989).

The fast, light-dependent degradation of the proteins that constitute the photosynthetic reaction center is unique to PSII and similar reactions do not occur in PSI or photosynthetic bacteria. In vivo, the lifetime of the D1 protein varies from 25 min (Greenberg et al., 1989) to a few hours (Kirilovsky et al., 1988) under different conditions. In addition, the degradation of the D1 protein (and to a lower extent the D2 protein) occurs also in vitro in thylakoid membranes (Virgin et al., 1988) and PSII enriched membranes (Virgin et al., 1990). This extensive degradation of the proteins that carry the components involved in PSII photochemistry is intriguing and obviously very expensive for the organism. Therefore, the understanding of the photochemical mechanism that triggers the damage to the reaction center proteins is crucial for our understanding of the funciton of PSII.

Although there is no kinetic correlation between the degradation of the D1 protein and the photoinhibition of the electron transfer through PSII (Virgin et al., 1988), there is a general agreement that the protein damage is caused by one of the photochemical reactions in PSII. Shortly after the discovery of the rapid turnover of the D1 protein, it was suggested that this was due to the accumulation of radical species in the Q_B site, which is located on the D1 protein (Kyle et al., 1984; Kyle, 1985). Thereafter, modifications of this hypothesis have been presented and it was recently suggested that the occupancy of the Q_B site controls the reaction (Critchley, 1988; Trebst et al., 1990). At variance with these models, it was proposed that the triggering reaction for the inhibition of the electron transfer (Cleland et al., 1986; Theg et al., 1986; Demeter et al., 1987) and the degradation of the D1 protein (Thompson & Brudvig, 1988; Styring et al., 1990) was the primary charge separation reaction or a donor side reaction. The arguments for the latter hypothesis (Styring et al., 1990) were based on the observation that, in photoinhibited thylakoids or PSII enriched membranes, the charge separation was still functional in a large fraction of the centers although the electron transport from pheophytin to Q_A was inhibited (Allakhverdiev et al., 1987; Vass et al., 1988; Styring et al., 1990). In addition, the idea was supported by the observation of increased D1 and D2 protein turnover as a consequence of illumination of hydroxylamine-washed chloroplasts (Callahan et al., 1986).

In this paper we test the hypothesis that the D1 degradation is due to donor side reactions in PSII. For this we study the degradation of the D1 and D2 proteins in Cl^--depleted thylakoids in which oxygen evolution is reversibly inhibited. In this material the photosynthetic electron transport, which can be reconstituted by Cl^-, is very sensitive to illumination (Theg et al., 1986) and we show that the D1 protein is rapidly degraded in this system.

Materials and Methods

Thylakoid membranes were prepared from spinach grown in a growth chamber at 475 μE m^{-2} s^{-1}. Control thylakoid membranes were prepared according to Andersson et al. (1976). The oxygen evolution of the control thylakoid membranes was about 200 μmol of O_2 (mg of chlorophyll)$^{-1}$ h^{-1}. Cl^--depleted thylakoid membranes were prepared with a modification of the method developed by Sandusky and Yocum (1989). Spinach leaves were homogenized in a Waring blender at the highest speed three times for 5 s in 25 mM HEPES–NaOH pH 7.8, 5 mM $MgSO_4$, 150 mM Na_2SO_4, and 400 mM sucrose (1 mL of buffer/g of leaves). The membranes were collected by centrifugation, washed twice in a large volume of 200 mM HEPES–NaOH, pH 7.5, and then resuspended (2 mL/g of leaves, resulting in an approximate concentration of 0.2 mg of chlorophyll/mL) in 200 mM HEPES–NaOH, pH 8.0, containing 6 μg of gramicidin/mL and incubated in this buffer for 30 min at 20 °C. The loss of activity during this incubation was lower than 10–15%, provided that the procedure was undertaken in darkness. After centrifugation the thylakoid membranes were washed once in a small volume of 20 mM HEPES–NaOH, pH 7.5, 5 mM $MgSO_4$, and 200 mM sucrose and then resuspended to the desired concentration in the same buffer. All preparation steps were performed in the dark or in very weak green light to avoid photodamage of the light-sensitive membranes. When the thylakoid membranes were not used directly, they were stored at −80 °C at a chlorophyll concentration of 3–5 mg of chlorophyll/mL.

Photoinhibition Experiments. Illumination of thylakoid membranes (100 μg of chlorophyll/mL) was performed aerobically at 20 °C in a thermostated reaction vessel, using continuous slow stirring. The sample was illuminated with heat-filtered white light from a 250-W projector lamp. The light intensity was varied with neutral-density filters and measured with a Skye Instruments quantum sensor. Photoinhibition was performed in 10 mM sodium phosphate buffer, pH 7.4, 100 mM sucrose, and either 5 mM $MgSO_4$ (in experiments performed in the absence of Cl^-) or 5 mM $MgCl_2$ + 50 mM NaCl. During the experiment samples were taken out at different time intervals for electron-transport assays. These were performed immediately after the sampling. Samples intended for Western blotting analysis were rapidly transferred and stored at −80 °C. Before they were frozen, 50 mM NaCl was added to the samples taken from experiments carried out in the absence of Cl^-. Some experiments were performed in the presence of 10 μM DCMU to inhibit the electron transfer at the acceptor side of PSII. In these experiments a separate sample was started for each point on the time course. For oxygen evolution measurements, the DCMU was removed after illumination by repeated centrifugation and resuspension of the sample in the dark at 0 °C. The washing was performed in the photoinhibition buffer

containing Cl^- to avoid further damage to the system during the procedure. This washing procedure resulted in efficient removal of the DCMU as revealed by control experiments in which nearly complete recovery of the oxygen evolution was achieved when DCMU-treated thylakoid membranes were washed.

Electron-Transport Assays. The oxygen evolution was measured in a Hansatech oxygen electrode by using saturating light. The assay medium was 25 mM HEPES–NaOH, pH 7.5, 2 mM $MgSO_4$, 40 mM NaCl, and 200 mM sucrose. When Cl^--depleted thylakoid membranes were assayed for their Cl^--dependent oxygen evolution the membranes were incubated for 2 min in the assay medium before the measurement to allow rebinding of Cl^- to its site. Phenyl-*p*-benzoquinone, 0.4 mM, was used as the external electron acceptor and was added from a 20 mM solution in dimethyl sulfoxide.

The electron transport from DPC to DCIP was measured in Tris-washed membranes at 590–540 nm in a Shimadzu UV3000 spectrometer equipped with sideways illumination. The assay medium consisted of 10 mM sodium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 50 mM NaCl, and 100 mM sucrose containing 1 mM DPC and 35 μM DCIP. DPC was prepared freshly during the experiment. To facilitate the electron donation from DPC, the photoinhibited samples were Tris-washed prior to the measurements in order to remove Mn and the water-soluble subunits in the oxygen-evolving complex (Andersson & Åkerlund, 1987). Tris washing was done in 0.8 M Tris-HCl at pH 8.2 for 20 min at 0 °C in room light. After they were Tris-washed the membranes were washed once in the photoinhibition medium containing Cl^- before the spectroscopic measurements.

Protein Analysis. SDS polyacrylamide gel electrophoresis was carried out as previously described (Ljungberg et al., 1986). Western blotting, with use of monospecific antibodies against the D_1, D_2, and 22-kDa proteins, was performed essentially according to Towbin et al. (1979), using ^{125}I-labeled protein A for detection. For quantification, the autoradiograms were scanned by a laser densitometer. In the analysis of the degradation of the D1 and D2 proteins, the 22-kDa protein in PSII was used as an internal standard, as this protein has been shown not to change markedly during photoinhibition (Virgin et al., 1990).

Results

Effect of Cl^- Depletion on the Light-Dependent Inhibition of Electron-Transfer Reactions in PSII. The Cl^--depleted thylakoid membranes used in this study had approximately 25% residual O_2 evolution when assayed in the absence of Cl^-. When the oxygen evolution was measured in the presence of 40 mM Cl^-, the activity was 85–90% of that in the control thylakoid membranes. Thus the Cl^- depletion affected 60–70% of the PSII reaction centers. All electron-transfer measurements presented below were done in the presence of 40 mM NaCl. Both the Cl^--depleted and the control thylakoid membranes were quite stable during the duration of the experiments. In 60 min less than 20% of the oxygen evolution activity was lost when Cl^--depleted membranes were kept in the dark at 20 °C in the absence of Cl^- (dark control to the experiments in Figures 1, 3, and 4). The control membranes were even more stable (dark control to Figure 2). The D1 protein was effectively insensitive to dark storage at 20 °C for several hours.

Figure 1 shows the time course for the photoinhibition of the oxygen evolution (closed circles) in the Cl^--depleted thylakoid membranes illuminated with light at 780 μE m^{-2} s^{-1}

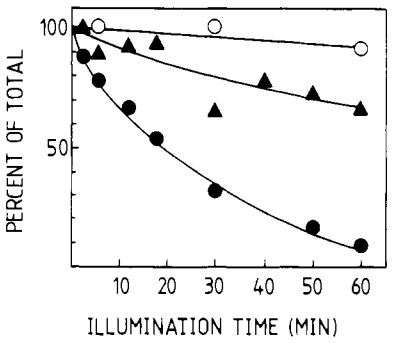


FIGURE 1: Time-dependent inhibition of oxygen evolution (closed symbols) and degradation of the D1 protein (open circles) in Cl^--depleted thylakoids that were illuminated with 780 μE m^{-2} s^{-1} either in the absence (closed circles) or in the presence of 60 mM Cl^- (closed triangles).

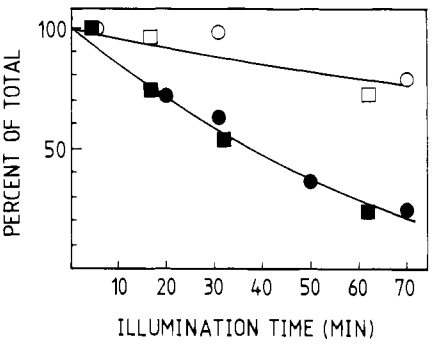


FIGURE 2: Time-dependent inhibition of the oxygen evolution (closed symbols) and degradation of the D1 protein (open symbols) in control thylakoid membranes illuminated with 6000 μE m^{-2} s^{-1}. The illumination was performed either in the absence (circles) or in the presence (squares) of 10 μM DCMU. The DCMU was removed prior to the oxygen measurements.

("low light"). The O_2 evolution was inhibited with a half-time of 18 min, showing monophasic kinetics. This should be compared to control thylakoid membranes in which less than 10% of the oxygen evolution was inhibited after 30-min illumination at this "low" light intensity. However, when control thylakoid membranes were illuminated at 6000 μE m^{-2} s^{-1} ("high light") the oxygen evolution was inhibited with a half-time of 35 min [Figure 2, closed circles; compare also Figure 1 in Virgin et al. (1988)]. In Cl^--depeleted thylakoid membranes the oxygen evolution was completely inhibited in less than 3 min at this light intensity. Thus, the oxygen evolution is 15–20 times more sensitive to illumination in the Cl^--depleted thylakoid membranes than in the control membranes. The light sensitivity of the Cl^--depleted membranes was also demonstrated in an experiment in which Cl^--depleted membranes were illuminated at different light intensities for a constant time of 10 min (Figure 3). The results show that the oxygen evolution (Figure 3A, closed circles) was totally inhibited in 10 min at light intensities above 3000 μE m^{-2} s^{-1} and that 50% inhibition occurred in 10 min at a light intensity of approximately 600 μE m^{-2} s^{-1}.

In the experiment presented in Figure 4 we compare the effect of photoinhibition of Cl^--depleted thylakoids on the electron transport from water to phenyl-*p*-benzoquinone (i.e., the oxygen evolution) and the electron transfer from DPC to DCIP, which measures the partial electron transfer from Tyr_Z (or Tyr_D, see Discussion) to the Q_B site. The results (Figure 4) show that the oxygen evolution (closed circles) is significantly more light sensitive than the electron transport from DPC to DCIP (open circles) at 1900 μE m^{-2} s^{-1}. The oxygen evolution was inhibited with a half-time of 4–5 min, while the DPC to DCIP electron transfer was inhibited with a half-time

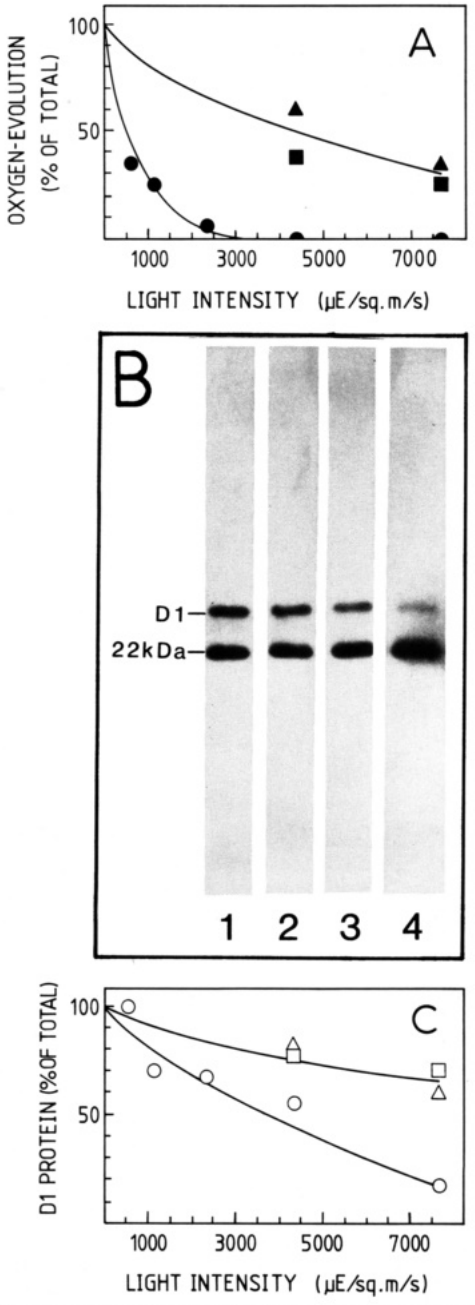


FIGURE 3: Light intensity dependent inhibition of the oxygen evolution and degradation of the D1 protein in Cl^--depleted thylakoid membranes that were illuminated for a constant time of 10 min with different light intensities. (A) Inhibition of the oxygen evolution. The illumination was performed in the absence of Cl^- or DCMU (circles), in the presence of 60 mM Cl^- (triangles) or in the presence of 10 μM DCMU (squares). When the photoinhibition experiment was performed in the presence of DCMU the herbicide was removed prior to the measurement of the oxygen evolution. (B) Immunoblot showing the levels of the D1 protein and the 22-kDa intrinsic protein after the experiment when Cl^--depleted thylakoid membranes were illuminated for 10 min with 625 μE m^{-2} s^{-1} (lane 2), 1170 μE m^{-2} s^{-1} (lane 3), or 7800 μE m^{-2} s^{-1} (lane 4). Lane 1 shows the level of the D1 protein in thylakoid membranes that were kept in the dark for 10 min at 20 °C in the absence of Cl^-. (C) Degradation of the D1 protein when the illumination of the Cl^--depleted thylakoid membranes was performed in the absence of Cl^- or DCMU (circles), in the presence of 60 mM Cl^- (triangles), or in the presence of 10 μM DCMU (squares). The quantification of the D1 protein was done by laser scanning densitometry of the immunoblot shown in (B) by using the 22-kDa protein as internal standard.

of 12–13 min (Figure 4, inset).

Effect of Cl^- Depletion on the Light-Dependent Degradation of the D1 and D2 Proteins. To observe the light-dependent

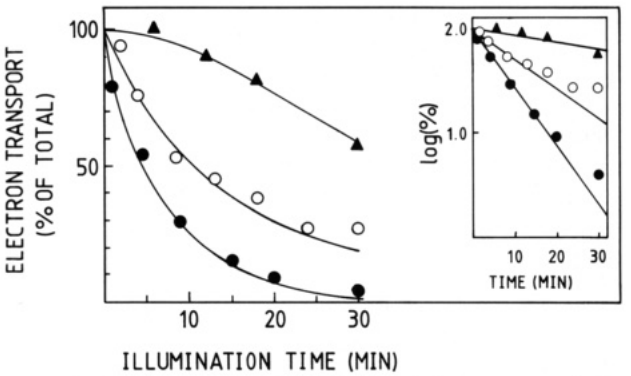


FIGURE 4: Time course for the inhibition of the oxygen evolution (closed symbols) and the electron transport from DPC to DCIP (open circles) in Cl^--depleted thylakoid membranes illuminated at 1900 μE m^{-2} s^{-1}. The illumination was performed either in the absence of Cl^- (circles) or in the presence of 60 mM Cl^- (triangles). The electron transport from DPC to DCIP was measured in Tris-washed, photoinhibited samples in the presence of 40 mM NaCl.

degradation of the D1 protein in control thlakoid membranes one is restricted to work at high light intensities and to illuminate the samples for extended periods of times (Virgin et al., 1988). Figure 2 shows the results of illumination of control thylakoid membranes with high light (6000 μE m^{-2} s^{-1}) and we estimate the half-time for the degradation of the D1 protein to 3 h (open circles).

In Cl^--depleted thylakoid membranes the situation is very different and at 6000 μE m^{-2} s^{-1} the half-time for the degradation of the D1 protein was about 8 min (not shown). In fact, the degradation of the D1 protein in the Cl^--depleted thylakoid membranes is so rapid that we could observe approximately 10% degradation of the D1 protein (Figure 1, open circles) after 60-min illumination at 780 μE m^{-2} s^{-1} ("low light"). Under these circumstances no D1 protein degradation can be observed in the control thylakoid membranes.

The degradation was further characterized in the light intensity experiments presented in Figure 3B,C. The results of the immunoblotting analysis of samples taken from Cl^--depleted thylakoid membranes illuminated with varying light intensities for 10 min are shown in Figure 3B. The band originating from the D1 protein decreases with increasing light intensity and at 7800 μE m^{-2} s^{-1} the D1 protein has almost disappeared (Figure 3B, lane 4). The quantitative data from the Western blot are presented in Figure 3C and it is seen that 85% of the D1 protein was degraded after a 10-min illumination at 7800 μE m^{-2} s^{-1}. In control thylakoid membranes a similar illumination protocol resulted in only 5–10% degradation of the D1 protein. We estimate that the D1 protein is at least 20 times more sensitive to illumination at 6000 μE m^{-2} s^{-1} in the Cl^--depleted thylakoid membranes. The lack in the Western blot (Figure 3B) of smaller fragments of the D1 protein cross-reacting with the antibody indicates that the degradation of the D1 protein in Cl^--depleted thylakoid membranes proceeds by a similar pathway as in control thylakoid membranes were the lack of observable fragments was earlier reported (Virgin et al., 1988).

The D2 protein was also followed in the light intensity experiment described in Figure 3. The data (not shown) indicate that the degradation of the D2 protein was enhanced as compared to the degradation in control thylakoid membranes in a comparable way to the degradation of the D1 protein but the D2 protein was not degraded to the same extent as the D1 protein [compare also with Figure 1 in Virgin et al. (1988)].

Chloride Protects against the Light-Dependent Inhibition

of the Electron Transport and the Degradation of the D1 Protein. Cl^- depletion is an essentially reversible process (Theg et al., 1984; Ono et al., 1986a,b) and by the readdition of Cl^- the oxygen evolution is almost completely recovered. We therefore tested whether the presence of Cl^- during illumination rendered Cl^--depleted thylakoid membranes less sensitive to the damaging effects of illumination. The results show that both the oxygen evolution (closed triangles in Figures 3A and 4) and the D1 protein (Figure 3C, open triangles) were 4–5 times more stable when the illumination was performed in the presence of 50 mM NaCl than in the absence of Cl^-.

Effects of DCMU on the Light-Dependent Inhibition of the Electron Transport and the Degradation of the D1 Protein. The herbicide DCMU, which inhibits the electron transfer through PSII, is reported to protect against photoinhibition (Kyle et al., 1984; Trebst et al., 1990), but the literature is ambiguous (Cleland & Melis, 1987) and it is not known whether DCMU can prevent the degradation of the D1 protein or not. Therefore, we tested the effect of DCMU on the photoinhibition of the oxygen evolution and the degradation of the D1 protein in both control thylakoid membranes and Cl^--depleted thylakoid membranes. In control thylakoid membranes, which were illuminated at 6000 μE m^{-2} s^{-1} ("high light"), DCMU did not protect the D1 protein against degradation (Figure 2, open squares) or the photosynthetic electron transport against photoinhibition (Figure 2, closed squares). However, in Cl^--depleted thylakoid membranes the effect of DCMU is very different. Figure 3A,C shows the results from the light intensity experiment. The presence of DCMU during the illumination efficiently prevented the degradation of the D1 protein in the Cl^--depleted thylakoid membranes (Figure 3C, open squares). In addition DCMU protected the oxygen evolution (Figure 3A, closed squares), in agreement with Theg et al. (1986). It should be noted that the herbicide was removed prior to the measurements of the oxygen evolution.

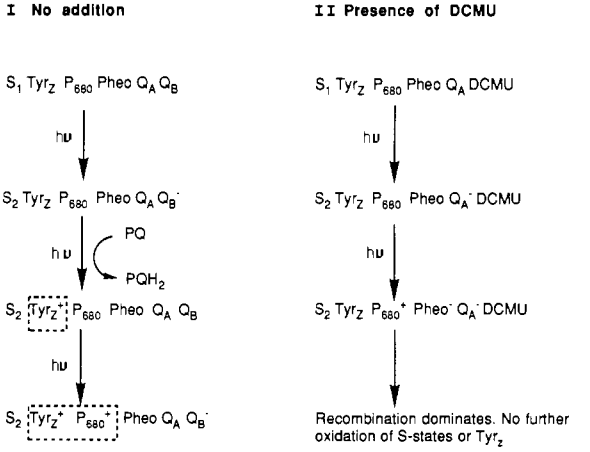
Discussion

To discuss the data it is necessary to introduce the photochemistry in PSII [for reviews, see Andréasson and Vänngård (1988) and Rutherford (1989)]. The absorption of a light quantum results in fast electron transfer from the primary electron donor P_{680} to the intermediary acceptor pheophytin and then to the first quinone acceptor, Q_A. From Q_A the electron is transferred to a second quinone, Q_B, which is bound to PSII in the semireduced state but exchanges with the plastoquinone pool in its oxidized or fully reduced state. The Q_B site is the action site for herbicides like DCMU that displace Q_B and thereby inhibit the forward electron transfer. P_{680}^+ is reduced from Tyr_Z and Tyr_Z^+ is reduced from the Mn cluster in the water-oxidizing complex. The water-oxidizing complex cycles between different oxidation states denoted S_n (n = 1–4). S_0 and S_1 are stable in the dark while S_2 and S_3 are more oxidized and deactivate to S_1 in the dark. In addition, environmental cofactors such as Cl^- and Ca^{2+} are needed for catalysis (Rutherford, 1989).

In the present study we have addressed the hypothesis that the degradation of the D1 protein is triggered by donor side reactions in PSII. This was done in photoinhibition experiments using Cl^--depleted thylakoid membranes. Cl^- depletion has been much studied and it is known that it results in a reversible inhibition of oxygen evolution due to a block in the S-cycle between S_2 and S_3 (Theg et al., 1984; Ono et al., 1986a,b).

The main conclusion from our measurements is that Cl^- depletion renders PSII very sensitive to photodamage also at light intensities where no light-induced damage is observed in control thylakoid membranes. Both the D1 protein and the oxygen evolution are 15–20 times more sensitive to illumination after the removal of Cl^-. The most likely explanation for this is that continuous illumination of Cl^--depleted thylakoid membranes induces abnormally long-lived, highly oxidizing radicals on the donor side in PSII. Scheme I (left) shows the reactions that lead to the accumulation of such species. In the absence of Cl^- the S_2 to S_3 transition is blocked. Thus, only one electron can be taken from the Mn cluster (which is oxidized from S_1 to S_2) and probably one more from Tyr_Z. Therefore only two normal reductions of P_{680}^+ can occur (Theg et al., 1984; Ono et al., 1986b). The next charge separation induces the radical pair P_{680}^+–Q_A^-, and, in the absence of a fast donor, the lifetime of the oxidized primary donor is increased to 40–50 μs (Ono et al., 1986b). After a short time both Tyr_Z^+ and P_{680}^+ can be anticipated to be present in anomalously high amounts. The acceptor side is functional in the Cl^--depleted system and thus the electron on Q_A^- is transferred to Q_B (Scheme I, left panel, lower reaction).

Scheme I[a]



[a] The left-hand panel shows the reactions that lead to the accumulation of oxidized species on the donor side of PSII in Cl^--depleted thylakoid membranes as a consequence of illumination. The right-hand panel shows how the presence of DCMU prevents the accumulation of oxidized species on the donor side of PSII when Cl^--depleted thylakoid membranes are illuminated.

In addition, the difference in the protective function of DCMU between photoinhibition of Cl^--depleted (Figure 3A,C) and control thylakoid membranes (Figure 2) is crucial for the understanding of the mechanisms that lead to photodamage of the D1 protein or to inhibition of the electron transfer. DCMU protected Cl^--depleted thylakoid membranes efficiently against the photoinhibition of electron transport and degradation of the D1 protein. This is explained in the right panel in Scheme I. In the presence of DCMU only one electron can be transferred to the acceptor side of PSII. The electron is taken from the Mn cluster, which is oxidized to the S_2 state. In Cl^--depleted thylakoid membranes this oxidation is allowed. Thereafter, the DCMU block prevents any further stable charge separations in the time course of the photoinhibition experiments with Cl^--depleted thylakoid membranes (Scheme I, right). The illumination continuously results in formation of the radical pair P_{680}^+–$Pheo^-$ but this recombines in a few nanoseconds in the presence of Q_A^- (Takahashi et al., 1987). This mechanism prevents the formation of long-lived, strongly oxidizing components on the donor side of PSII (that were formed in the absence of DCMU in Cl^--depleted thylakoid membranes) and protects in this way both the electron-transport reactions and the D1 protein against photo-

damage. In principle, strong illumination could lead to overreduction of the acceptor side as in control thylakoid membranes (Styring et al., 1990). However, these reactions occur with a very low quantum yield (probably even lower in the case of Cl^- depletion since there is no efficient charge storage system) and are not expected to take place during the much shorter illumination times applied in the experiments with the Cl^--depleted thylakoid membranes.

In control thylakoid membranes, illuminated with high light, DCMU did not protect the electron transport or the D1 protein against photodamage (Figure 2). Under these light conditions it is easy to explain the lack of effect of DCMU. In strong light Q_A^- will dominate in the acceptor complex in the presence of DCMU. However, it is likely that prolonged illumination results in the accumulation of reduced pheophytin, which eventually reduces Q_A a second time. The reactions are essentially similar to those proposed to take place during strong illumination in the absence of DCMU (Styring et al., 1990). Under these circumstances (intact thylakoid membranes illuminated with high light intensitites) the inhibition of the oxygen evolution is due to a block in the electron transfer from pheophytin to Q_A probably caused by double reduction of Q_A (Styring et al., 1990). In the inhibited centers the primary charge separation reaction was still functional, which led to the suggestion that the degradation of the D1 protein was triggered by the charge separation reaction or donor side reactions in the perturbed centers. These reactions are not influenced by the presence of DCMU, which thus explains the lack of DCMU protection against degradation of the D1 protein (Figure 2).

In Cl^--depleted thylakoid membranes [Figure 4; compare also Figure 1f in Theg et al. (1986)] the oxygen evolution is inhibited three times faster than the electron transfer from DPC to DCIP, which was inhibited with a half-time of 12–13 min at 1900 $\mu E\ m^{-2}\ s^{-1}$. From the data in Figure 3C we have calculated an approximate half-lifetime for the D1 protein of 15–16 min at the same light intensity. Thus the D1 protein is degraded with nearly similar kinetics as the inhibition of the DPC–DCIP electron transfer. This indicates that the inhibition of the DPC to DCIP electron transport might be directly correlated to the *primary* protein damage of the reaction center.

Normally DPC is considered to donate electrons to Tyr_Z (Yerkes & Babcock, 1980), which is located on the D1 protein (Debus et al., 1988b; Metz et al., 1989). If this was the case our results imply that the oxygen evolution initially was inhibited between the Mn cluster and Tyr_Z and that the DPC donation later was inhibited at or after Tyr_Z, possibly together with the initial step in the protein damage. This model is reasonable but recent evidence indicates that other explanations might be available (Blubaugh & Cheniae, 1990). It was suggested that photoinhibition of hydroxylamine-treated chloroplasts rapidly damaged Tyr_Z. This activated a secondary, more stable, electron donation site, which was proposed to be Tyr_D, an accessory donor on the D2 protein (Debus et al., 1988a; Vermaas et al., 1988). Later this site also became inactivated by the illumination. The results are analogous to ours since they imply two levels for the photoinhibition of the electron transport. If the interpretations are applicable to our results, the data on the photoinhibition in Cl^--depleted thylakoids can be readily explained. The illumination leads to the accumulation of long-lived oxidizing species on the donor side of PSII (Scheme I, left). This inhibits the electron transfer via Tyr_Z and thus the oxygen evolution measured after Cl^- replenishment (Figure 4). At the same time P_{680}^+, which still must be functional since the electron transfer from DPC to DCIP occurs, starts to take electrons from nearby components. In the presence of DPC the electron transfer could occur via Tyr_D (Blubaugh & Cheniae, 1990) but in the absence of DPC the oxidized form of Tyr_D is stable (Mathis & Rutherford, 1987). The lack of other endogenous or exogenous electron donors to P_{680}^+ then increases the lifetime of the oxidized primary donor.

From these considerations we cannot identify the component responsible for the initial protein damage, but our results reduce the candidates to two, P_{680}^+ or Tyr_Z^+, which both are highly oxidizing and potentially hazardous for the protein environment. The oxidizing side of PSII works at redox potentials much higher than encountered in other photosynthetic systems. The midpoint potential of P_{680} is about 1.1 V (Klimov et al., 1979), which is 650 mV more oxidizing than P_{870} in purple bacteria (Parson, 1987) and 700 mV higher than P_{700} in PSI (Sêtif & Mathis, 1980). In addition Tyr_Z^+ is strongly oxidizing (Metz et al., 1989). In Cl^--depleted thylakoid membranes the illumination results in the accumulation of P_{680}^+ and Tyr_Z^+ (Scheme I). It is not probable that the mere presence of Tyr_Z^+ or P_{680}^+ induces large tertiary rearrangements in the structure of the D1 protein, since they are continuously formed in the normal light reactions. When the donor side of PSII functions well, P_{680}^+ is reduced from Tyr_Z in 20–200 ns (Brettel et al., 1984). However, when Tyr_Z^+ is inactivated or remains oxidized, P_{680}^+ becomes long-lived (Ono et al., 1986a). Under these circumstances it is highly likely that P_{680}^+ (and possibly also Tyr_Z^+), due to its high oxidative potential, can oxidize nearby amino acid residues or close-lying redox components (Thompson & Brudvig, 1988), which might destroy the tertiary structure of the D1 protein. The modified D1 polypeptides are then excised from the reaction centers and further degraded in proteolytic reactions to prevent the accumulation of nonfunctional PSII units in the thylakoid membranes (Mattoo et al., 1989; Virgin, et al., 1990). It is probable that these secondary proteolytic reactions are very efficient, since we did not observe the accumulation of any large degradation products in the Cl^--depleted thylakoid membranes, although the D1 protein was degraded quite rapidly (Figure 3B). Moreover, recent results suggest that the polypeptides from PSII that are not degraded are reutilized in the reassembly of new, functional PSII centers together with newly synthesized D1 proteins (Virgin et al., 1990).

The question is then to what extent these results and considerations, derived from experiments with Cl^--depleted thylakoid membranes, are applicable to the situation in vivo, where the D1 protein turnover is rapid also at moderately high light. The data presented here and our earlier measurements in intact thylakoid membranes (Virgin et al., 1988; Styring et al., 1990) indicate that donor side reactions are responsible for triggering the degradation of the D1 protein (other mechanisms may apply for the inhibition of the electron transport, see below), and we suggest that the same mechanism applies also in vivo. In this model, the most likely situation that triggers the degradation of the D1 protein is a slowing down of the electron transfer to P_{680}^+, which could result from inhibition of the water-splitting cycle or inactivation of Tyr_Z. Possible candidates in the plant for lesions leading to such effects on the donor side of PSII include the freezing-induced loss of the 23-kDa extrinsic protein, involved in the water-splitting reaction (Wang et al., 1990) (in vivo this would lead to a situation reminiscent of Cl^- depletion), decreased efficiency of the S-state transitions at high light due to slow release of O_2 from the system (Plijter et al., 1988), or heat-induced damage to

the water-splitting complex (Cheniae & Martin, 1970; Nash et al., 1985). Another possibility is the UV-induced inactivation of Tyr_Z that was recently observed by EPR spectroscopy [Yerkes et al., 1990; see also Greenberg et al. (1989)]. The UV spectrum for the photosensitizer responsible for the degradation of the D1 protein presented by Greenberg et al. [Grenberg et al., 1989; see also Jones and Kok (1966)] was interpreted as being due to plastosemiquinone. However, this interpretation can be questioned, since the spectrum is somewhat similar also to the difference spectrum of Tyr_Z/Tyr_Z^+ (Dekker et al., 1984; Diner & DeVitry, 1984).

It was suggested that the photoinactivation of Cl^--depleted and Tris-washed thylakoids was mechanistically the same phenomenon as photoinhibition in intact systems (Theg et al., 1986). This is not the case, as it has been conclusively shown that high light photoinhibition of intact systems is due to limitations on the acceptor side of PSII (Arntz & Trebst, 1986; Allakhverdiev et al., 1987; Ohad et al., 1988; Vass et al., 1988; Styring et al., 1990). On the other hand, it is clear that systems inhibited in the water oxidation are sensitive to illumination due to donor-side limitations (Theg et al., 1986; Callahan et al., 1986). Thus, several possibilities exist for the inhibition of electron transport through PSII. This might explain differences in the protective effects of DCMU-type herbicides in studies performed in vivo. At our low-light conditions, which resemble the light conditions often applied in photoinhibition experiments using in vivo systems, we suggest that D1 turnover and inhibition of the electron transport are quite closely correlated and caused by side reactions due to the formation of oxidizing species on the donor side of PSII. In this situation, DCMU-type inhibitors will protect the system. However, at extremely high light, the donor side is more efficient than the acceptor side. The latter becomes overreduced, which inhibits the electron transfer. The D1 protein is damaged in later reactions that are not DCMU sensitive. Thus, herbicides will not protect PSII under these conditions. In this case, it is not unlikely that the protein damage, in part, is caused by the formation of singlet oxygen (Masojidek et al., 1990). This is the case for the D1/D2/cytochrome b_{559} preparation, which is very light sensitive in the presence of oxygen but much more stable in its absence (He et al., 1990). This preparation lacks Q_A and is in this respect quite similar to the photoinhibited PSII centers in which the function of Q_A is impaired.

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Kinetic Analysis of Cooperative Interactions Induced by Mn^{2+} Binding to the Chloroplast H^+-ATPase†

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ABSTRACT: The kinetics of Mn^{2+} binding to three cooperatively interacting sites in chloroplast H^+-ATPase (CF_1) were measured by EPR following rapid mixing of the enzyme with $MnCl_2$ with a time resolution of 8 ms. Mixing of the enzyme-bound Mn^{2+} with $MgCl_2$ gave a measure of the rate of exchange. The data could be best fitted to a kinetic model assuming three sequential, positively cooperative binding sites. (1) In the latent CF_1, the binding to all three sites had a similar on-rate constants of $(1.1 \pm 0.04) \times 10^4$ M^{-1} s^{-1}. (2) Site segregation was found in the release of ions with off-rate constants of 0.69 ± 0.04 s^{-1} for the first two and 0.055 ± 0.003 s^{-1} for the third. (3) Addition of one ADP per CF_1 caused a decrease in the off-rate constants to 0.31 ± 0.02 and 0.033 ± 0.008 s^{-1} for the first two and the third sites, respectively. (4) Heat activation of CF_1 increased the on-rate constant to $(4.2 \pm 0.92) \times 10^4$ M^{-1} s^{-1} and the off-rate constants of the first two and the third site to 1.34 ± 0.08 and 0.16 ± 0.07 s^{-1}, respectively. (5) The calculated thermodynamic dissociation constants were similar to those previously obtained from equilibrium binding studies. These findings were correlated to the rate constants obtained from studies of the catalysis and regulation of the H^+-ATPase. The data support the suggestion that regulation induces sequential progress of catalysis through the three active sites of the enzyme.

The H^+-ATPase from chloroplasts utilizes an electrochemical potential of protons for the synthesis of ATP from ADP and P_i (McCarty & Carmeli, 1982). Its catalytic sector (CF_1)[1] is a complex having a subunit stoichiometry of 3α, 3β, γ, δ, and ϵ (Morony et al., 1983). Subunit interaction is a major regulatory process in the function of the H^+-ATPase of chloroplasts as it is in similar enzymes from other sources such as mitochondria and bacteria. Negative cooperativity in nucleotide binding is accompanied with a faster catalysis (Cross, 1981), while positive cooperativity in the binding of divalent metal ions is accompanied with a slow down in pre-steady-state catalysis (Carmeli et al., 1981). In accordance with earlier proposals, Stroop and Boyer (1987) suggested that equivalent interactions of the three catalytic β subunits occur as they

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CF_1, coupling factor 1 of chloroplast H^+-ATPase; EBT, Eriochrome black T.

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