

Stepwise Photoinhibition of Photosystem II¹

Studies with *Synechocystis* Species PCC 6803 Mutants with a Modified D-E Loop of the Reaction Center Polypeptide D1

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Several mutant strains of *Synechocystis* sp. PCC 6803 with large deletions in the D-E loop of the photosystem II (PSII) reaction center polypeptide D1 were subjected to high light to investigate the role of this hydrophilic loop in the photoinhibition cascade of PSII. The tolerance of PSII to photoinhibition in the autotrophic mutant $\Delta R225-F239$ (PD), when oxygen evolution was monitored with 2,6-dichloro-*p*-benzoquinone and the equal susceptibility compared with control when monitored with bicarbonate, suggested an inactivation of the Q_B -binding niche as the first event in the photoinhibition cascade in vivo. This step in PD was largely reversible at low light without the need for protein synthesis. Only the next event, inactivation of Q_A reduction, was irreversible and gave a signal for D1 polypeptide degradation. The heterotrophic deletion mutants $\Delta G240-V249$ and $\Delta R225-V249$ had severely modified Q_B pockets, yet exhibited high rates of 2,6-dichloro-*p*-benzoquinone-mediated oxygen evolution and less tolerance to photoinhibition than PD. Moreover, the protein-synthesis-dependent recovery of PSII from photoinhibition was impaired in the $\Delta G240-V249$ and $\Delta R225-V249$ mutants because of the effects of the mutations on the expression of the *psbA-2* gene. No specific sequences in the D-E loop were found to be essential for high rates of D1 polypeptide degradation.

Prolonged strong irradiation impairs the photochemical efficiency of all oxygen-evolving photosynthetic organisms. PSII, an intricate thylakoid membrane complex with a light-driven water-plastoquinone-oxidoreductase activity, is the primary target of this phenomenon, which is known as photoinhibition (see Prasil et al., 1992; Aro et al., 1993). Despite intense research, the molecular mechanism of PSII photoinhibition in vivo still remains unclear. On the basis of in vitro studies, two major pathways have been implicated in the photodamage of PSII. The acceptor-side photoinhibition, typical under strong illumination, occurs at the level of the primary quinone electron acceptor Q_A , which leaves its site in the D2 polypeptide after being double reduced (Styring et al., 1990; Vass et al., 1992). Such conditions lead to the recombination of the primary radical pair $P680^+ Pheo^-$ and to the formation of chlorophyll triplets (Vass et al., 1992). Triplet chlorophyll may then react with molecular oxygen to produce toxic singlet oxygen, which damages PSII. The other mechanism, donor-

side photoinhibition, takes place when the donation of electrons to PSII occurs more slowly than their removal to the acceptor side, leading to the formation of long-lived, highly oxidizing radicals such as Tyr Z^+ and $P680^+$ (Blubaugh and Cheniae, 1990; Jegerschöld et al., 1990; Bumann and Oesterheld, 1995). These species have the capacity to extract electrons from their surroundings, causing irreversible damage to PSII. Depending on environmental conditions, both of these photoinhibition mechanisms have been implicated in in vivo photoinhibition as well (Wang et al., 1992; Ohad et al., 1994). Studies conducted with intact cells suggest an initial role for the Q_B pocket in the induction of PSII photoinhibition (Kyle et al., 1984; Kirilovsky et al., 1988; Ohad et al., 1990).

It is generally agreed that light-induced damage to PSII renders the D1 polypeptide ready for degradation. Detrital oxidative species produced by PSII may induce a conformational change in D1, thereby making it susceptible to proteolysis (see Prasil et al., 1992; Aro et al., 1993). Restoration of PSII function following photoinhibitory damage and subsequent degradation of the damaged D1 polypeptide requires de novo synthesis and the incorporation of a new D1 copy into the PSII complex (Prasil et al., 1992; Aro et al., 1993).

Although the mechanism of D1 degradation is not yet known, the D-E loop of this polypeptide is thought to have an important regulatory role in the process of D1 polypeptide degradation. The D-E loop not only forms an essential structural component of the Q_B pocket (Trebst, 1986), but also has specific amino acid sequences, the PEST-like sequence and the putative cleavage region (Greenberg et al., 1987), which are postulated to carry information for D1 polypeptide degradation. We have recently constructed *Synechocystis* sp. PCC 6803 mutants with large deletions in the D-E loop of the D1 polypeptide, and have shown that the electron transfer on the acceptor side of PSII is severely disturbed in these mutants (Mulo et al., 1997). The deletion of the PEST-like sequence in the autotrophic strain PD impaired the Q_A -to- Q_B electron transfer in PSII, whereas deletions in the putative cleavage region of the D1 polypeptide in CD and PCD also resulted in perturbation of the quinone-exchange reaction in the Q_B pocket, preventing photosynthetic growth (Mulo et al., 1997).

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Abbreviations: AR, antibiotic-resistant strain; CD, $\Delta G240-V249$ mutant; DCBQ, 2,6-dichloro-*p*-benzoquinone; DMBQ, 2,5-dimethyl-*p*-benzoquinone; PCD, $\Delta R225-V249$ mutant; PD, $\Delta R225-F239$ mutant.

In the present study the mutants described above were employed to investigate how the severe modifications of the Q_B pocket and consequent perturbations in the function of the acceptor side of PSII modulate the susceptibility of PSII to photoinactivation and how they exert their effect on the subsequent repair process.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

D1 polypeptide mutants, which are derivatives of the Glc-tolerant strain of *Synechocystis* sp. PCC 6803 (Williams, 1988), were constructed as described by Mulo et al. (1997). The mutant strains PD, CD, and PCD were maintained on BG-11 plates supplemented with antibiotics (see Mäenpää et al., 1993), 5 mM Glc, and 10 μ M DCMU to prevent any selective advantage of possible revertants or secondary mutants. The only functional *psbA* gene in all mutants was the mutated *psbA-2*, whereas *psbA-1* and *psbA-3* genes were inactivated by interruption with antibiotic-resistance cassettes (Mohamed and Jansson, 1989).

All strains were grown under a continuous PPFD of 40 μ mol $m^{-2} s^{-1}$ at 32°C. The AR control, which contains the same antibiotic-resistance cassettes as the mutants but no mutations in the *psbA-2* gene, and the autotrophic mutant PD were grown as described in Mäenpää et al. (1993), except that 5 mM Glc was included during growth on the plates. Glc was omitted from the liquid medium to allow photoautotrophic growth (the growth mode of the cells did not affect the results obtained). The heterotrophic strains CD and PCD were grown on plates like those used for AR and PD, but the liquid growth medium was supplemented with 5 mM Glc.

In Vivo High-Light Treatments

Synechocystis sp. PCC 6803 cells were harvested (4500g, 10 min, room temperature) at the logarithmic growth phase and resuspended in BG-11 medium to a final concentration of 10 μ g chlorophyll mL^{-1} . The cells were illuminated at a PPFD of 500, 1000, or 1500 μ mol photons $m^{-2} s^{-1}$ as indicated, using a slide projector as a light source. Temperature was controlled so as not to exceed 32°C, and aliquots for various measurements were withdrawn every 15 min. After 60 min of incubation at high light, cells were transferred back to growth light (40 μ mol photons $m^{-2} s^{-1}$) and the recovery of the cells from photoinhibition was monitored by measuring the oxygen-evolving capacity after 0, 1, 2, 3, and 4 h. In some experiments, as indicated, the translation inhibitor lincomycin (400 μ g mL^{-1}) was added immediately after the high-light treatment, prior to the transfer of the cells to recovery conditions.

In Vivo Pulse-Chase Experiments and Determination of D1 Protein Half-Life

In vivo pulse-chase experiments were performed as described previously (Tyystjärvi et al., 1994). Before high-light treatment, L -[^{35}S]Met (in vivo cell-labeling grade, 1000

Ci $mmol^{-1}$, Amersham) was added to the cell suspension (10 μ g chlorophyll mL^{-1}) to a final concentration of 1.2 μ M. The cells were pulse-labeled for 75 min at 1500 μ mol photons $m^{-2} s^{-1}$, unlabeled Met (1 mM) was added, and thereafter the cells were washed and resuspended in fresh BG-11 medium supplemented with 1 mM cold Met. Radioactivity was chased for 0, 15, 30, 45, and 60 min at high light (1500 μ mol photons $m^{-2} s^{-1}$).

Photosynthetic membranes were isolated and polypeptides were separated as described previously (Tyystjärvi et al., 1994) by using 12% SDS-polyacrylamide gels including 4 M urea (Laemmli, 1970). Solubilized membranes containing 2 μ g of chlorophyll were loaded into each well. After electrophoresis, gels were treated with Amplify (Amersham), dried, and exposed to radiographic film. Fluorograms were scanned with a laser densitometer, and the half-life of the D1 polypeptide was determined by fitting the data to the first-order equation.

In Vivo Measurement of Oxygen Evolution

Oxygen evolution was measured under saturating red light with a Clark-type oxygen electrode (Hansatech, Kings Lynn, UK) at 32°C with a slide projector as the light source. Before measurements, 1 mL of cell suspension corresponding to 10 μ g chlorophyll mL^{-1} was harvested and resuspended in fresh BG-11 medium. Either 0.5 mM DCBQ or 1.0 mM DMBQ was used as the artificial electron acceptor, and 0.25 mM ferricyanide was added to keep the quinones in oxidized form. The light-saturated rate of oxygen evolution was also measured by supplementing the growth medium with 0.6 mM bicarbonate.

Isolation of RNA and Northern Blotting

Cell samples were collected after 0, 15, 30, 45, and 60 min of high-light treatment (1500 μ mol photons $m^{-2} s^{-1}$), and the total RNA was isolated using the hot phenol method in the presence of 60 mM EDTA and 0.5% SDS (Sherrer and Darnell, 1962). Northern analysis was performed using the standard methods (Sambrook et al., 1989) described in detail by Tyystjärvi et al. (1996). Membranes were first probed with the entire *psbA-2* gene of *Synechocystis* sp. PCC 6803, and then the membrane was stripped and reprobed with *rrn* genes of *Anacystis nidulans* (Tomioka et al., 1981) to verify equal loading of the gel and even transfer of RNA in blotting.

Chlorophyll Determination

The chlorophyll *a* content of the cell suspensions and isolated membranes was determined according to the methods of Bennett and Bogorad (1973) and Arnon (1949), respectively.

RESULTS

Oxygen Evolution Rates and Inhibition of Electron Transport by DCMU in the Mutant Strains

The D-E loop deletion mutants of the D1 polypeptide, PD, CD, and PCD, showed striking differences in the

oxygen-evolving capacity with various electron acceptors (Table I). The autotrophic PD and control AR strains, as well as the heterotrophic strains CD and PCD, were all capable of electron transfer from water to DCBQ, which has been suggested to accept electrons directly from Q_A (Graan and Ort, 1986). In a slight variance from our earlier results (Mulo et al., 1997), all D-E loop mutants, including PD, were severely perturbed in electron transfer to DMBQ, which accepts electrons from Q_B , probably by interacting with the Q_B site (Graan and Ort, 1986). Despite low-DMBQ-mediated oxygen-evolution activity, the PD mutant exhibited nearly as high a rate of oxygen evolution as the control strain AR when no artificial electron acceptors were present.

We also tested the ability of DCMU to replace different electron acceptors in the Q_B pocket. The addition of 1 μM DCMU completely inhibited the DCBQ-dependent oxygen-evolution activity in the control AR cells, but had no effect in the mutants PD, CD, and PCD. However, a minor inhibition of DCBQ-dependent oxygen evolution was observed in the mutants if the DCMU concentration was increased to 10 μM . DMBQ- and bicarbonate-mediated oxygen evolution, by contrast, were completely blocked by 1 μM DCMU, both in the control AR and in the mutants PD and CD. Only in the PCD strain was a higher DCMU concentration (10 μM) required to totally inhibit the oxygen evolution from water to DMBQ (Table I).

Susceptibility of PSII to Photoinhibition in the D-E Loop-Deletion Strains of *Synechocystis* sp. PCC 6803

Upon illumination at a PPF of 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the autotrophic PEST-deletion strain PD was much more resistant to photoinhibition of PSII than the control strain AR or the heterotrophic strains CD and PCD when assayed with DCBQ after the high-light treatment (Fig. 1A). Light treatment at 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ resulted in an even more drastic difference between AR and PD; more than 80% of activity was lost in AR, indicating that the PPF of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was still strong enough to

induce severe inactivation of PSII oxygen evolution, whereas only a 30% loss was observed in PD after 60 min of illumination (Fig. 1B). To exclude the possibility that the PD cells with low PSII activity were already partially photoinactivated during the growth period at 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, both AR and PD cells were grown at much lower light intensity, below 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and no variance to the results above was observed (data not shown).

The cleavage-region mutants CD and PCD, which exhibited similar kinetics of photoinactivation under a PPF of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (as did the control strain AR [Fig. 1A]), were somewhat more resistant to photoinhibition than AR under a PPF of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, but were still clearly more susceptible than PD (Fig. 1B). Notably, however, when PSII activity was measured with DMBQ (Fig. 1C) or bicarbonate (Fig. 2B) as the electron acceptor, the mutants were as susceptible to photoinhibition as AR. Unlike the mutant strains, no significant differences were detected in the susceptibility of the AR cells to photoinhibition whether DCBQ or DMBQ was used as the artificial quinone electron acceptor to monitor the progress of photoinhibition (Fig. 1, B and C).

Because of rapid photoinactivation of the AR cells at 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, we further lowered the light intensity to measure the intrinsic susceptibility of PSII photoinactivation in the presence of the protein-synthesis inhibitor lincomycin, which prevents the concurrent repair of PSII. Distinct differences prevailed in the susceptibility of PSII to photoinhibition between AR and PD, even when protein synthesis was blocked; PD was clearly more tolerant to photoinhibition than the control strain AR when measured with DCBQ (Fig. 1D). Under similar conditions, however, no difference could be detected in the rate of photoinhibition between the AR and PD strains if oxygen evolution was assayed with bicarbonate as the final electron acceptor; a severe photoinhibition of PSII was now also seen in the PD strain (Fig. 1D).

Recovery from Photoinhibition

The results above indicate that PSII in the PD cells is intrinsically more tolerant against photoinhibition when assayed with DCBQ, whereas a very similar sensitivity of the AR and PD cells was revealed if oxygen evolution was monitored with bicarbonate (Fig. 1D). To elucidate this apparent controversy, we illuminated the AR and PD cells at 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and then transferred the cells to low recovery light with the simultaneous addition of the protein-synthesis inhibitor lincomycin to prevent protein-synthesis-dependent repair of PSII centers. The differential susceptibility of PSII to high light in the AR and PD cells was evident when oxygen evolution was monitored with DCBQ (Fig. 2A), whereas equal susceptibility was recorded when bicarbonate-dependent oxygen evolution was monitored (Fig. 2B).

After the transfer of cells to low light in the presence of lincomycin, practically no changes in oxygen-evolution activity took place in the PD or AR cells when monitored with DCBQ (Fig. 2A) or in AR cells monitored without the

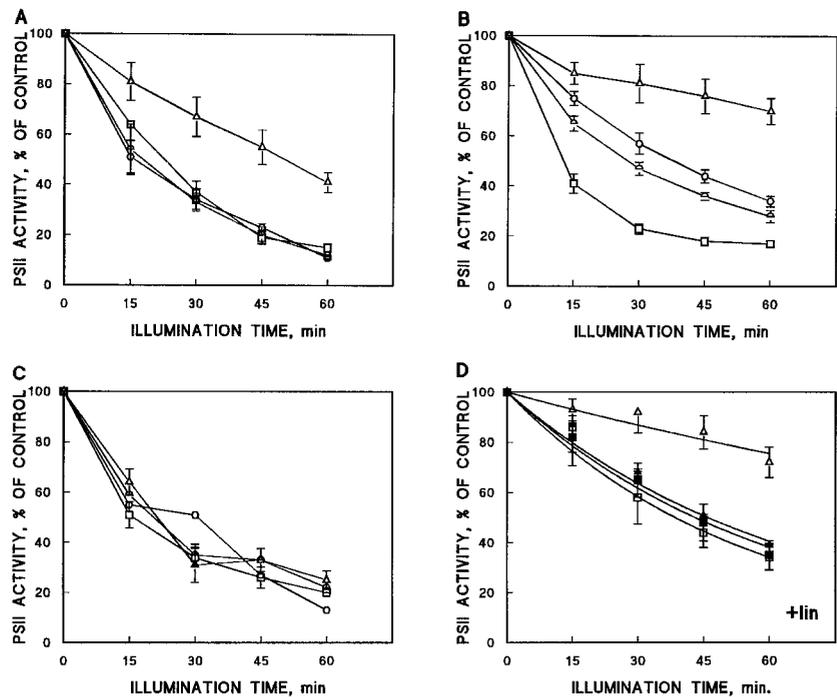
Table I. Oxygen-evolution rates in *Synechocystis* sp. PCC 6803 control and mutant strains measured with different electron acceptors, and the inhibition of oxygen evolution by DCMU

Susceptibility of oxygen evolution to inhibition by 1 μM DCMU is given in parentheses: +, complete inhibition of oxygen evolution; +/-, partial inhibition of oxygen evolution, and -, no or only a minor effect of 1 μM DCMU. Results are the means \pm SE of 3 to 10 independent experiments from different cell cultures.

Strain	Oxygen Evolution		
	DCBQ	DMBQ	Bicarbonate
	$\mu\text{mol O}_2 \text{ mg}^{-1} \text{ Chl}^a \text{ h}^{-1}$		
AR	231 \pm 18 (+)	260 \pm 39 (+)	160 \pm 15 (+)
PD	91 \pm 8 (-)	34 \pm 2 (+)	136 \pm 9 (+)
CD	146 \pm 15 (-)	32 \pm 2 (+)	Trace (+)
PCD	148 \pm 15 (-)	40 \pm 4 (+/-)	Trace (+)

^a Chl, Chlorophyll.

Figure 1. Photoinactivation of PSII oxygen evolution during illumination of AR (\square) and mutant cells (PD, \triangle ; CD, \circ ; and PCD, \ominus) of *Synechocystis* sp. PCC 6803. Results are the means of three to five independent experiments from different cell cultures, and are expressed as a percentage of oxygen-evolution activity measured before the high-light treatment. Bars denote SE. A, Cells were illuminated at a PPFD of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and oxygen evolution was measured in vivo using DCBQ as the electron acceptor. B, Cells were illuminated at a PPFD of $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and oxygen evolution was assayed in vivo with DCBQ as the electron acceptor. C, Cells were illuminated at a PPFD of $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and oxygen evolution was assayed in vivo with DMBQ as the electron acceptor. D, The PD and AR cells were illuminated at a PPFD of $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the presence of lincomycin ($400 \mu\text{g/mL}$) and oxygen evolution was monitored with DCBQ (\square , \circ) or with bicarbonate (\blacksquare , \bullet) as the electron acceptor.



addition of artificial electron acceptor (Fig. 2B). In sharp contrast to AR, nearly 50% restoration of bicarbonate-dependent oxygen evolution, with no concurrent protein synthesis, took place in the PD cells during 1 h of incubation at low light (Fig. 2B). Taken together, these results strongly suggest that photoinhibition in the PD strain, when monitored with bicarbonate, was comprised of two components, one irreversible and one reversible, whereas DCBQ-mediated oxygen-evolution measurements only recorded the irreversible photoinhibitory component of PSII.

To follow the protein-synthesis-dependent restoration of PSII oxygen evolution (water-DCBQ) in photoinhibited cells, all strains were first illuminated for 60 min at a PPFD of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. This illumination induced severe (about 80%) photoinhibition in all strains except PD; the PSII activity of the PD cells remained somewhat higher despite several trials to enhance photoinhibition either by prolonged incubation of the cells at $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ or by increasing the light intensity up to $2500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (data not shown). Despite severe photoinhibition, the transfer of the control AR and the mutant strains PD and CD back to growth-light conditions induced full restoration of their PSII function within 4 h (Fig. 2C). This restoration of PSII function was fully dependent on protein synthesis in all strains (data not shown). It should be noted that during the 1st h of recovery, more than 50% restoration of the PSII activity occurred in AR and PD cells, whereas the cleavage-region mutants recovered more slowly, with less than 20% restoration occurring within the 1st h. The overall capacity of the PCD strain to recover from photoinhibition during 4 h of incubation was clearly suppressed compared with the other strains (Fig. 2C).

Degradation Rates of the D1 Protein at High Light

The half-lives of the D1 protein in AR and the mutant strains at a PPFD of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured with the pulse-chase technique are given in Table II. Half-lives at growth conditions (Mulo et al., 1997) are also given for comparison. In AR the half-life of the D1 polypeptide at high light was 26 min, whereas in all mutants the half-life was clearly longer: 45 min for PD, 56 min for CD, and 53 min for PCD. Figure 3 shows examples of pulse-chase fluorograms for the mutant strains PD, CD, and PCD, the two latter strains lacking the putative cleavage region (Greenberg et al., 1987). Thylakoid proteins of the heterotrophic strains CD and PCD were more heavily labeled than those of the autotrophic strain PD because of the faster growth rate of the cells, which was induced by Glc in the growth media of CD and PCD. In addition to pulse-chase experiments, monitoring the degradation of the D1 polypeptide by immunoblotting during the high-light treatment of cells in the presence of the protein-synthesis inhibitor lincomycin also revealed a significantly longer half-life for all mutant strains compared with the AR control strain (data not shown).

Variation of the *psbA-2* Transcript Level during Photoinhibitory Illumination

The fluctuation in the amount of the *psbA-2* mRNA, the product of the only active *psbA* gene in all of the strains, was followed in the course of photoinhibitory illumination. In the AR strain, the amount of the *psbA-2* mRNA at growth-light conditions was low, but the high-light treatment induced a strong accumulation of the *psbA-2* mRNA (Figs. 4 and 5). PCD also had a low level of the *psbA-2*

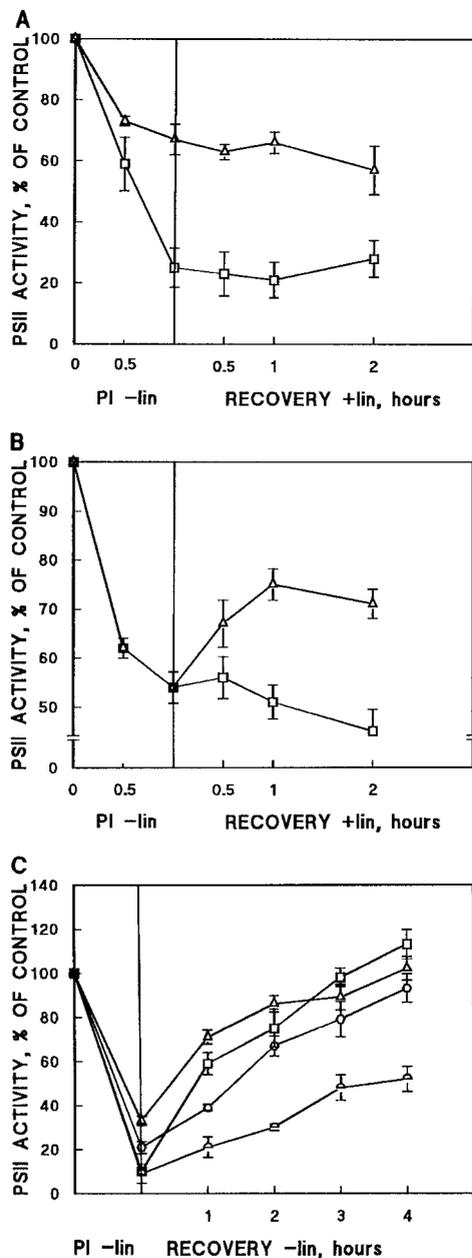


Figure 2. Recovery of PSII from photoinhibition. Protein-synthesis-independent (A and B) and protein-synthesis-dependent (C) restoration of PSII oxygen evolution in *Synechocystis* PCC 6803 strains after photoinhibitory illumination. Results are means of three independent experiments from different cell cultures and bars denote SE. A, AR (\square) and PD (\triangle) cells were illuminated at $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 60 min (PI) in the absence of lincomycin. The subsequent recovery of oxygen evolution was followed in the presence of lincomycin at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and was monitored with DCBQ as the electron acceptor. B, The AR (\square) and PD (\triangle) cells were illuminated at $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 60 min (PI) in the absence of lincomycin. The subsequent recovery of oxygen evolution at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the presence of lincomycin was monitored using bicarbonate as the electron acceptor. C, AR (\square), PD (\triangle), CD (\circ), and PCD (∇) strains were illuminated at $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 60 min (PI), and the restoration of DCBQ-dependent oxygen evolution was followed at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. All treatments were performed in the absence of lincomycin.

mRNA at growth-light conditions, but, unlike the AR strain, no drastic accumulation of the *psbA-2* mRNA occurred during high-light illumination. In the PD and CD strains the steady-state level of the *psbA-2* mRNA under growth-light conditions was about 10-fold higher than that in the AR and PCD strains (Mulo et al., 1997), and remained at a high level during the high-light illumination of 60 min. In the presence of the protein-synthesis inhibitors lincomycin (Figs. 4 and 5) and chloramphenicol (data not shown), only a slight increase in the amount of the *psbA-2* transcripts was detected at the beginning of the high-light illumination. However, this declined back to the growth-light level during the 60-min high-light treatment.

DISCUSSION

We have constructed a unique autotrophic PSII mutant with a deletion in the D-E loop of the D1 polypeptide (Mulo et al., 1997). This deletion has significantly modified the conformation of the Q_B pocket, as evidenced by a strongly modified oxygen-evolution capacity with different artificial electron acceptors and the incapability of DCMU to inhibit DCBQ-dependent oxygen evolution (Table I). Apparently because of these drastic modifications in the Q_B site, the PD mutant showed specific tolerance against photoinactivation of PSII when assayed with DCBQ, which has been suggested to accept electrons directly from Q_A (Graan and Ort, 1986; Satoh et al., 1993, 1995) (Figs. 1, A, B, and D, and 2A). Since the yield of variable fluorescence was similar in the presence and absence of DCBQ, it can be concluded that in the PD mutant, reduced Q_A also serves as the electron donor to DCBQ (E. Tyystjärvi, personal communication), thereby excluding the possibility that the mutation had created new avenues for the reduction of DCBQ. No specific tolerance of the PD cells to high light was evident when photoinhibition of oxygen evolution was assayed with DMBQ (Fig. 1C) (there were, however, extremely low activities; Table I) or with bicarbonate as the final electron acceptor (Fig. 2B). Nevertheless, even this inhibition differed from that of the con-

Table II. Half-life of the D1 polypeptide in the site-specific D-E loop mutants of *Synechocystis* sp. PCC 6803 at high-light and growth-light conditions

Cells were pulse labeled with $L\text{-}[^3\text{S}]\text{Met}$ and the radioactivity was chased at either high-light ($1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) or growth-light ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) conditions. Fluorograms were analyzed with a laser densitometer, and the results were fitted to first-order kinetics to determine the half-life ($t_{1/2}$) of the D1 polypeptide. Results are the means \pm SE of two to four independent experiments.

Strain	Half-Life	
	High light	Growth light ^a
	<i>min</i>	
AR	26 ± 3	198 ± 18
PD	45 ± 3	120 ± 6
CD	56 ± 2	108 ± 18
PCD	53 ± 5	162 ± 18

^a Data were taken from Mulo et al. (1997).

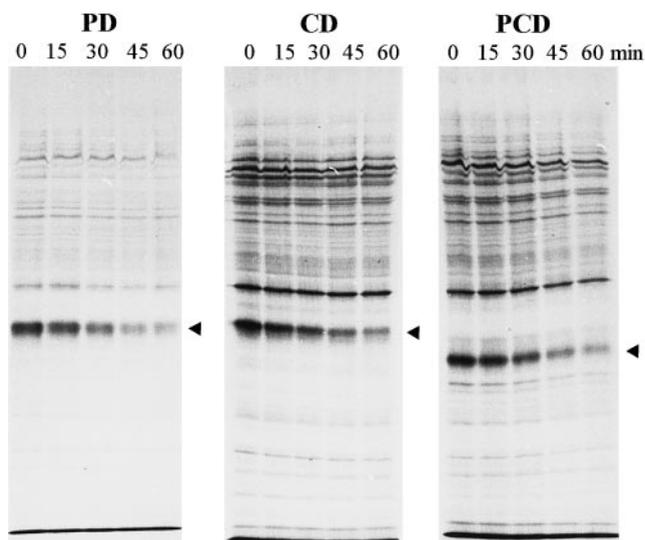


Figure 3. Chase of radioactivity in membrane polypeptides of *Synchocystis* sp. PCC 6803 mutants. The cells were first pulse labeled for 75 min in the presence of L-[³⁵S]Met, and then radioactivity was chased for 0, 15, 30, 45, and 60 min at 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Arrowheads indicate the location of the mutated D1 polypeptides. Correct identification of the radiolabeled D1 polypeptide was confirmed by western blotting.

trol AR cells in being largely reversible, without the need for de novo protein synthesis, when the cells were transferred to low-light conditions (Fig. 2B).

The photoinhibitory behavior of the PD mutant has important consequences for our understanding of the mechanism of PSII photoinhibition in vivo. These results imply that the process of PSII photoinhibition can be dissected into at least two relatively independent but probably sequential phases. The initial light-induced modification of the Q_B -binding site in the PD mutant appeared to be a reversible event, with no requirement for protein synthesis, as far as the inhibition had not progressed to an inhibition of Q_A reduction (Fig. 2B). The latter event, in contrast, was irreversible in all strains (Fig. 2A, data not shown for CD

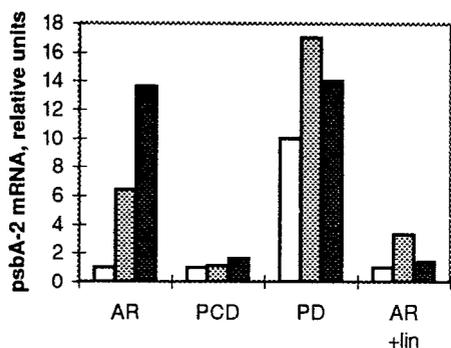


Figure 4. Accumulation of the *psbA-2* mRNA in the AR, PCD, and PD cells in the course of high-light illumination (1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in the absence or presence of lincomycin, as indicated. The amount of RNA is given in arbitrary units comparable between the strains. White bars, 0 min of illumination; stippled bars, 30 min of illumination; and black bars, 60 min of illumination.

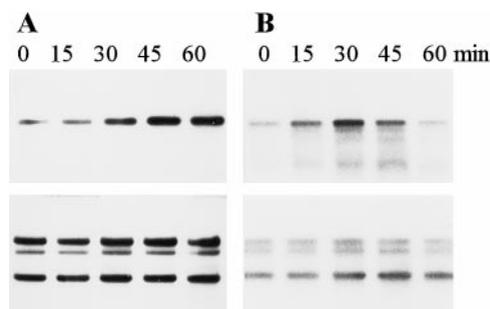


Figure 5. Representative northern blots showing the *psbA-2* mRNA levels in the AR strain during photoinhibitory illumination of 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the absence (A) and presence (B) of the protein-synthesis inhibitor lincomycin. The bottom panels present the same membranes probed with *rrn* genes of *A. nidulans*. Five micrograms of total RNA was loaded in each well.

and PCD). Such a reversible, high-light-induced initial modification of the Q_B pocket could not be trapped in the AR control strain, probably because of a short lifetime.

The initial role of the Q_B pocket in PSII photoinactivation in vivo was suggested in very early photoinhibition studies (Kyle et al., 1984) and later with the ΔPEST ($\Delta\text{E226-Q233}$; Nixon et al., 1995) and ΔpsbH (Komenda and Barber, 1995) mutants of PSII. The reversibility of this reaction in vivo was deduced from fluorescence and thermoluminescence experiments (Kirilovsky et al., 1990; Ohad et al., 1990), but a clear biochemical demonstration for such a reversible conformational change in the Q_B pocket under physiological conditions has been missing until now. It is conceivable that the initial modification in the Q_B -binding site renders the cells susceptible to subsequent irreversible events: an inhibition of Q_A reduction and a consequent irreversible damage to the D1 protein. Generally, the reversible phase is difficult to trap (Vavilin et al., 1995) because the subsequent irreversible phases rapidly follow the initial conformational change in the Q_B pocket. This model of early photoinhibitory events in the Q_B pocket agrees with the reduced rate of PSII photoinactivation at low temperatures reported in several systems both in vivo and in vitro (Aro et al., 1990; Kirilovsky et al., 1990), presumably because the initial conformational changes in the Q_B pocket slow down with declining temperatures.

What could be the molecular basis for such a unique response of the PD cells to photoinhibitory illumination? The ΔPEST mutant (Nixon et al., 1995), which has a somewhat smaller deletion in the same region of the D-E loop compared with that of PD, showed no such tolerance against high-light-induced photoinhibition. Detailed comparison of these two autotrophic mutants ΔPEST and PD, both showing perturbations in the function of the acceptor side of PSII, also revealed an important difference. Both thermoluminescence and fluorescence-decay measurements point to a very small driving force for the electron transfer from Q_A^- to Q_B in the PD mutant (Mulo et al., 1997), whereas ΔPEST is much less severely affected in this respect (Nixon et al., 1995). It is conceivable that the large deletion in PD, including amino acids of the possible contact site between the D1 and D2 proteins (Trebst and

Soll-Bracht, 1996), also drastically modifies the Q_A -binding site in the D2 protein, rendering Q_A^- less accessible to reoxidation.

Generally, the mutants with modified acceptor-side function of PSII, including herbicide-resistant mutants carrying point mutations in the Q_B -binding site (Kirilovsky et al., 1989; Constant et al., 1996), the *psbH* deletion mutant (Mayes et al., 1993; Komenda and Barber, 1995), the Δ PEST mutant (Nixon et al., 1995), and the strains with point mutations or small deletions in the PEST and cleavage-site regions (Tyystjärvi et al., 1994), have been reported to be intrinsically either equally or more sensitive to light-induced damage of PSII than the wild type. No simple correlation, however, seems to exist between the efficiency of electron-transfer reactions on the acceptor side of PSII and the susceptibility of PSII to photoinhibition. Nevertheless, a picture is now emerging suggesting that mutations that mainly alter the Q_B -binding properties may render the cells more susceptible to photoinhibition, whereas mutations that lead to an isolation of Q_A^- from Q_B may protect PSII centers against irreversible photodamage. PD belongs to the latter group, whereas the heterotrophic CD and PCD strains also suffer from extremely poor exchange of plastoquinone in the Q_B pocket (Table I; Mulo et al., 1997), which exacerbates the irreversible photoinhibitory damage to PSII.

PSII photoinactivation was accompanied by D1 protein degradation in all strains. Although the amino acid stretches in the PEST-like sequence and in the putative cleavage region probably carry no specific information for light-dependent D1 protein degradation, the overall changes in the conformation of the loop are likely to modulate the degradation of the D1 protein. Thus, all mutant strains exhibited somewhat slower D1 degradation at high light than the AR control strain (Table II). In the PD mutant this is probably related to slower irreversible inactivation of PSII centers, whereas a distorted conformation of the D-E loop is likely to retard D1 degradation in CD and PCD compared with the AR cells. Our results also question a signal from the Q_B site to the degradation of the D1 protein upon illumination of the cells (Bracht and Trebst, 1994; Komenda and Barber, 1995).

As demonstrated with the PD mutant, the degradation of the D1 protein is not directly related to the initial conformational change in the Q_B -binding site that renders the cells incapable to reduce plastoquinone. This reaction indeed seems to be a reversible event with no need for D1 protein replacement (Fig. 2B). Only the subsequent event, inactivation of electron transfer from water to Q_A , was clearly also an irreversible process in PD (Fig. 2A), and this inhibition is likely to give a signal for degradation of the D1 polypeptide.

The retarded D1 degradation in the D-E loop deletion mutants under strong illumination seems to contradict results obtained under growth-light conditions, which showed accelerated D1 protein turnover compared with the AR control strain (Table II). However, it is conceivable that under growth-light conditions the mutated and misfolded D1 polypeptides are efficiently degraded by non-specific, "household" protease(s), whereas under high ir-

radiances such "cleaning" is masked by an efficient function of a still unknown D1-specific proteinase.

Slow initial protein-synthesis-dependent recovery of the heterotrophic mutants CD and PCD from photoinhibition (water to DCBQ) compared with the AR and PD strains (Fig. 2C) agrees with the measured low degradation capacity of the photodamaged D1 polypeptide in these mutants (Table II). The final recovery of the CD cells, however, was as complete as that of the control AR strain, whereas the PCD mutant, with almost similar susceptibility to photoinhibition as the CD cells, showed only poor capacity for PSII repair (Fig. 2C). Since degradation of the D1 polypeptide from photoinhibited centers is equally retarded in the CD and PCD mutants, this difference in the recovery capacity possibly points to impaired D1 synthesis in PCD.

The wild type (Mohamed and Jansson, 1989; Kanervo et al., 1993) and control AR and the mutants PD and CD possessed high amounts of *psbA-2* transcripts at high light, whereas PCD was the only mutant not able to efficiently accumulate *psbA-2* mRNA at high light (Figs. 4 and 5). A similar situation could be mimicked in the AR cells by blocking the protein synthesis with lincomycin during the high-light treatment. This might infer a link between regulation of the *psbA-2* gene transcription and the D1 protein synthesis and suggests that there are interacting factors in the regulation of efficient D1 turnover rather than a single determinant such as the light intensity.

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