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Reversible and irreversible photoinhibition in herbicide-resistant mutants of *Synechocystis* 6714

Diana Kirilovsky, Claudie Vernotte, Chantal Astier and Anne-Lise Étienne

C.N.R.S., Laboratoire de Photosynthèse (E.R. 307), Gif-sur-Yvette (France)

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The behaviour of two herbicide-resistant mutants of *Synechocystis* 6714, DCMU-II$_B$ and Az-V, were compared to the wild type during various times of exposure to high light intensity (photoinhibition). The kinetics of the loss of variable fluorescence were similar in the three strains. However, Az-V cells lost the ability to recover Photosystem II activity more rapidly than wild-type and DCMU-II$_B$ cells. Radiolabeling experiments showed that the turnover of D_1 is similar in wild type and Az-V. Partial reactions of electron flow through Photosystem II were measured on thylakoids isolated from cells withdrawn at different times of photoinhibition. The decrease of oxygen evolution using DCBQ (electron acceptor after Q_B), presented the same kinetics in wild type and Az-V. In contrast, the kinetics of decrease of oxygen evolution with silicomolybdate were faster in Az-V than in wild type. Our results support the hypothesis that the Q_B site of reaction center II is the initial target of damage by photoinhibition. This damage can be reversed by de novo synthesis of D_1 and D_2 proteins. The reversible inhibition is followed by a more extensive degradation of the core complex RC II. This more extensive degradation is irreversible and is characterized by a decrease of energy transfer from the phycobilisomes to the Photosystem II, and incapacity to perform charge separation. Due to a higher instability of their core complex II the second, irreversible step of degradation happens more rapidly with Az-V mutant cells than with wild-type cells.

Introduction

The exposure of photosynthetic organisms to high light intensities results in a decline of CO_2 fixation and photosynthetic oxygen evolution (Photoinhibition (PI)) [1,2]. The primary lesion is observed in Photosystem II (PS II) (reviewed in Refs. 3 and 4). During photoinhibition the variable fluorescence (F_v) and PS II activity decrease. Synthesis of thylakoid proteins is necessary to recover this activity.

Controversial theories about the primary site of photodamage were proposed these last years. The results of some laboratories [5–10] seemed to indicate that the inactivation of the reaction center II (RC II) is the primary event in photoinhibition, but Kyle et al. [11] have suggested that initially only the Q_B protein (D_1) is damaged.

Present theory needs to take into account the new model of PS II. Nowadays, it is accepted that the RC II is composed by the cytochrome *b*-559 and the heterodimer D_1/D_2 which carries the P-

Abbreviations: DCBQ, dichlorobenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PI, photoinhibition; PS II, Photosystem II; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; RC II, reaction center II; SiMo, silicomolybdate.

Correspondence: D. Kirilovsky, C.N.R.S., Laboratoire de Photosynthèse (E.R. 307), 91198 Gif-sur-Yvette Cedex, France.

680, I, Z, D and the primary (Q_A) and secondary (Q_B) quinones [12–15]. From similarities of the bacterial reaction center with the complex D_1/D_2, it was proposed that the Q_B and herbicides are bound inside a niche formed by the hydrophylic loop of D_1 (on the stroma side) which links the helical segments IV and V which span the membrane. The D_1 protein serves other functions as well, e.g., His-198 in helix IV is probably involved in the binding of chlorophyll P-680 [14].

In the present stage of knowledge on the composition and structure of Photosystem II, it might prove useful to study photoinhibition in herbicide-resistant mutants with a modified D_1 protein.

In this report, the behaviour under photoinhibition stress of a wild strain of *Synechocystis* 6714 was compared with that of two mutant strains with a high relative resistance to one herbicide (DCMU or atrazine). We found that while our DCMU resistant cells behave like wild-type cells, the atrazine-resistant cells are more sensitive to light illumination.

The results obtained indicate that the binding site of Q_B was modified in a first step, which was followed by a more extensive damage of the D_1/D_2/cytochrome *b*-559 complex that led to an irreversible inactivation of PS II.

Materials and Methods

Growth conditions. Wild-type and mutants cells of *Synechocystis* 6714 were grown in the mineral medium described by Herdman et al. [16] with twice the concentration of nitrate and an illumination of about 20 W/m^2. Other conditions were as previously described [17].

Thylakoid membranes preparations. Thylakoid membranes were isolated by a modification of the method described by C. Astier et al. [18]. Cells (100–200 μg Chl/ml) were suspended in 15 mM Hepes (pH 6.8), 30 mM $CaCl_2$, 25% glycerol (v/v) solution (buffer A) with 0.46 mg/ml of glass beads and were broken by three disruptions of 15 s at the highest speed in a MSK cellular homogenizer. Unbroken cells and glass beads were removed by centrifugation at $3000 \times g$ for 5 min. Subsequently, the supernatant was centrifuged at $120\,000 \times g$ for 10 min. The pellets were resuspended in a minimal volume of buffer A and stored at $-80\,^\circ C$ until use. Most of the phycobilisome proteins are released but the phycobilisome-linker protein of 95 kDa molecular mass remains attached to the membranes.

Photoinhibition experiments. Photoinhibition of cell suspensions (30 μg Chl/ml) was carried out at $25\,^\circ C$. The light intensity was about 1200 W/m^2 provided by four Atralux spots of 150 W. For recovery from photoinhibition, the cells were centrifuged, resuspended in fresh growth medium and incubated for 3 h at low light illumination (like in normal cell growth).

SDS-polyacrylamide gel electrophoresis. Analysis of thylakoid polypeptide pattern was performed, after incubation at $40\,^\circ C$ for 30 min for denaturation, using the method of Laemmli [19]. The electrophoretic separation was carried out using SDS and a polyacrylamide gradient of 10%–17% in the presence of 4 M urea. The gel was stained by Coomassie blue, dried and autoradiographed.

Spectroscopy and measurements of photosynthetic activities. Fluorescence kinetics were performed using whole cells as previously described [18]. Low-temperature fluorescence spectra were recorded using a home-built apparatus described by C. Astier et al. [18]. Photosynthetic activity measurements were carried out polarographically as oxygen evolution using SiMo [20] or DCBQ as electron acceptor for PS II activity.

Flash-induced reduction of Q_A was monitored at 410 nm and 445 nm [21]. These wavelengths are zero-crossing points of the P-700 difference spectrum, so that Q_A reduction is monitored with minimal interference by PS I. The thylakoids were resuspended in buffer A containing TMPD (250 μM), ferricyanide (40 μM) and DCMU (1 μM) to a concentration of 25 μg Chl/ml. A sample was illuminated (in anaerobic conditions) by a short saturating flash; this was repeated 32 times using dark intervals of 1 min, and the absorbance change recorded at 200 ms after the flash was averaged. A correction for remaining PS I interference was applied by subtracting the result obtained when the measurement was repeated using dark intervals of 2 s; the 2-s intervals are sufficient for rereduction of P^+-700 but insufficient for reoxidation of Q_A^-.

Chlorophyll concentrations were determined according to Bennet and Bogorad [22].

Results

Two mutants of *Synechocystis* 6714 were chosen for photoinhibition studies. The first one DCMU-II_B is resistant to DCMU but not to atrazine. The second one is resistant to atrazine, but not to DCMU. They have the same rate of electron transfer from Q_A to Q_B, which is only slightly decreased compare to the wild type [17].

Variable fluorescence measurements

Decrease of variable fluorescence in the presence of DCMU is commonly used to follow the time-course of photoinhibition [11]. Fig. 1 shows that the rate of loss of variable fluorescence was the same in wild type, DCMU-II_B and Az-V cells exposed to high light. Half time of decrease was about 10–12 min and kinetics are apparently first order.

After 30, 60 and 90 min of high light, cells were resuspended in fresh medium and incubated in low light during 3 h. Wild-type and DCMU-II_B cells which have been exposed to high light for 30 and 60 min, respectively, completely regained their initial variable fluorescence. In contrast, Az-V cells recuperate their initial F_v after 30 min of photoinhibition, but not after 60 min. All three strains were unable to recover after 90 min of photoinhibition (data not shown). These results suggest that Az-V cells reach a state of irreversible PS II

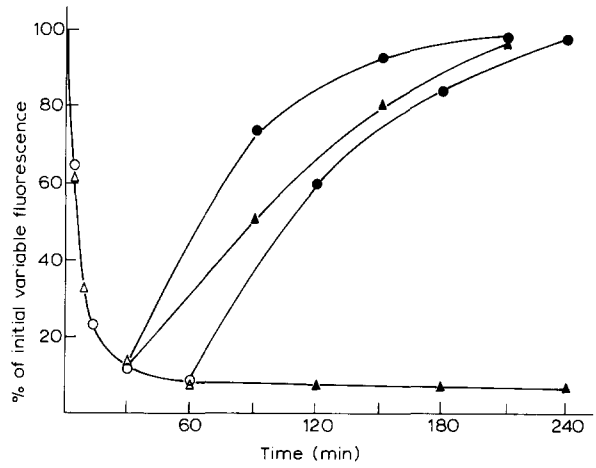

Fig. 1. Loss (○, △) and recovery (●, ▲) of variable fluorescence $F_v = (F_m - F_o)/F_o$ in wild-type (○, ●) and Az-V mutants (△, ▲) of *Synechocystis* 6714. Cell suspensions (1 μg/Chl per ml) were excited at 440 nm in the presence of DCMU (10^{-5} M). 100% of F_v is equal to 1–1.5. During an hour of photoinhibition F_0 decreased only 15%. The kinetics of variable fluorescence in DCMU-II_B mutant are similar to those of wild-type cells. Cells of all strains photoinhibited during 90 min were unable to recover any variable fluorescence.

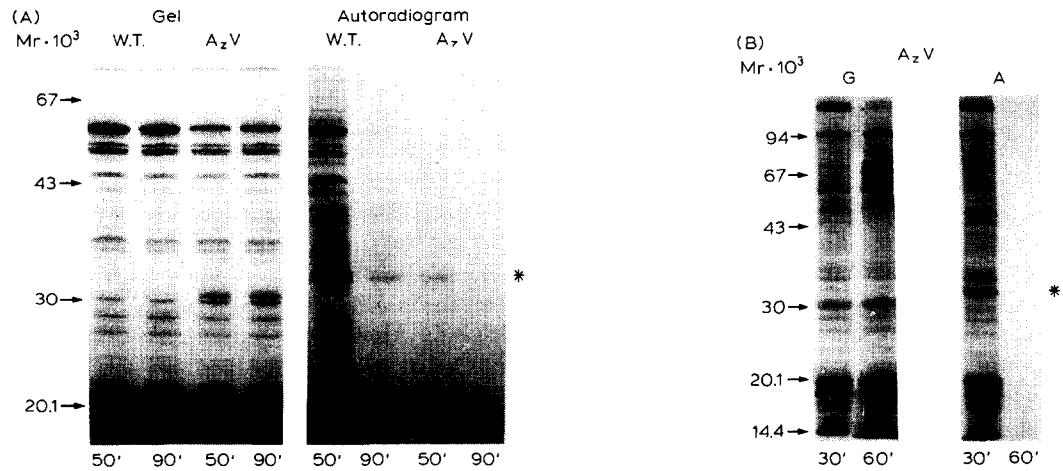

Fig. 2. Pulse-labeling of wild-type and Az-V mutant cells during recovery from photoinhibition. The cells were photoinhibited for (A) 50 min and 90 min, and (B) 30 and 60 min, and then they were allowed to recover in the presence of $^{35}SO_4^{2-}$. The thylakoids were isolated after 3 h of cell incubation in dim light. G, gel; A, autoradiogram. The asterisk indicates the location of the D_1 protein.

inactivation faster than wild type and DCMU-II_B cells. Radiolabeling experiments supported this hypothesis.

Radiolabeling experiments

Protein synthesis during recovery in normal conditions (20 W/m²) after photoinhibition. Cells which have been photoinhibited were centrifuged and incubated in growth medium devoid of $MgSo_4$

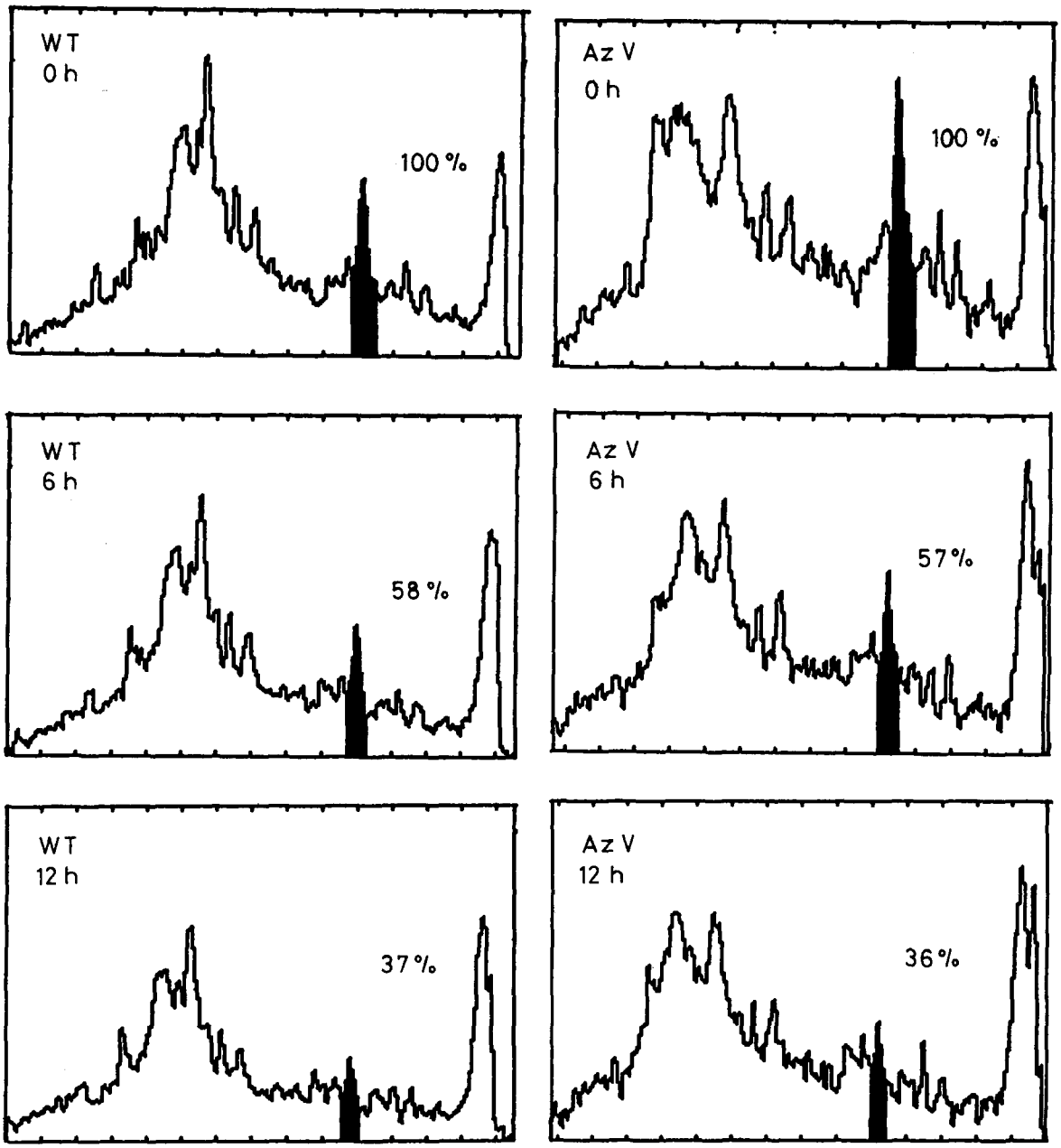

Fig. 3. Densitometry scan of autoradiograms showing the turnover of D_1. Wild-type and Az-V cells (30 μg Chl/ml) were incubated in growth conditions with $^{35}SO_4^{2-}$ during 90 min. After addition of non-radioactive SO_4^{2-} samples were taken at 0, 6 and 12 h. The black peak locates D_1. 100% of the radioactivity in D_1 is equal to 7% of the total radioactivity.

and containing $^{35}SO_4^{2-}$ (4 μCi/ml) during 3 h in low light at 30 μg Chl/ml. Thylakoids were then extracted; SDS-polyacrylamide gel electrophoresis and autoradiograms were performed. Fig. 2A shows that wild type cells photoinhibited during 50 min and allowed to recover during 3 h in dim light, have incorporated ^{35}S into the thylakoids proteins and especially in the 32–34 kDa bands which correspond to D_1 and D_2 molecular weights. The same is true for Az-V cells photoinhibited during 30 min (Fig. 2B), but not for all the other conditions, i.e., wild type cells photoinhibited during 90 min and Az-V cells photoinhibited for 50, 60 or 90 min, conditions that, according to Fig. 1 correspond to an incapacity to recover variable fluorescence. These results establish a correlation between the appearance of irreversible PS II inactivation and a loss of the capacity to synthetize new proteins.

Turnover of thylakoid proteins in normal growth conditions ($I = 20\ W/m^2$). The observed difference between the wild type and Az-V strain in their response to photoinhibiting conditions could be due to different protein turnover, so we measured it in normal conditions. Washed cells were resuspended in growth medium without $MgSO_4$ at a chlorophyll concentration of 30 μg/ml in the presence of $^{35}SO_4^{2-}$. After 2 h of incubation, cold $MgSO_4$ (10 mM) was added. Thylakoids were isolated at 0, 6 and 12 h and analyzed by gels and autoradiograms. Fig. 3 shows scans of the obtained autoradiograms. In wild type at time 0, 7% of the thylakoid radioactivity was in the Q_B protein. About 73% of the initial radioactivity was lost from this protein after 12 h of chase. During this time, only 20% of the total membranal proteins were degraded, indicating the faster turnover of the D_1 protein. The same results were obtained for Az-V cells, therefore the distinct behaviour of Az-V cells is not due to a different turnover of proteins.

Fluorescence emission spectra at 77 K

Fluorescence emission spectra at 77 K of wild-type and Az-V mutant cells were measured before and after exposure to high intensity illumination for 1 h (Fig. 4). The spectra of both strains before photoinhibition are characterized by four peaks with their maxima at 650, 660, 690 and 720 nm corresponding to phycocyanin, allophycocyanin, allophycocyanin B (max 686 nm) + PS II (max 696 nm), and PS I, respectively. In both cases the peak at 690 nm shifted to 686 nm and the ratio 696 nm/720 nm decreased during photoinhibition, showing a specific loss of PS II fluorescence. Photosystem I fluorescence remained the same before and after exposure to high light illumination. However, while the peak ratio 686 nm/720 nm has decreased in wild-type cells it has increased in Az-V cells after 60 min of photoinhibition. We did not observe any changes in the absorbance spectra of these samples, neither at 682 nm (chlorophyll peak) nor at 628 nm (phycocyanin peak) (data not shown). Then no bleaching of chlorophyll occurred in the cells during photoinhibition. These results indicate that energy

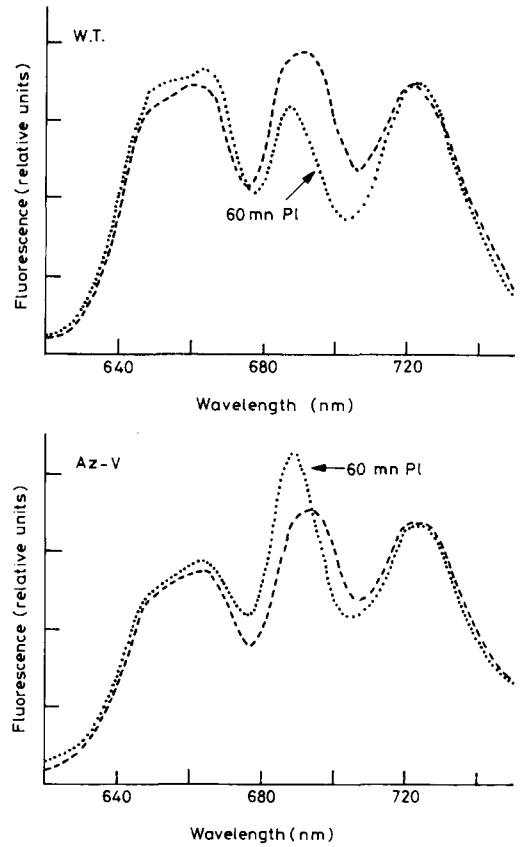

Fig. 4. Fluorescence emission spectra at 77 K of wild-type and Az-V mutant cells before (— — —) and after (· · · · · ·) exposition to high light during 1 h. Excitation wavelength: 560 nm ($\Delta\lambda = 15$ nm); emission $\Delta\lambda = 3$ nm.

transfer from the phycobilisomes to Chl *a* has become less effective in Az-V cells after 60 min of photoinhibition. The same result was obtained in the wild-type cells if the time of exposure to high light intensity was prolonged, and in that case, the recovery was also inhibited (data not shown).

It seems that during the reversible step of photoinhibition the phycobilisomes remain attached to the thylakoids and the energy transfer to Chl *a* is normal. However, during the irreversible step a dissociation of phycobilisomes from the membranes may occur as a result of extensive damage to the RC II.

Electron transfer through Photosystem II

Partial reactions of the electron transfer through PS II were utilized to define the primary target of photoinhibition. We have chosen to measure oxygen evolution with two different acceptors: DCBQ and SiMo. The first one accepts electrons from PS II in presumably the same way as Q_B, so the integrity of the Q_B site is necessary. The second acceptor, SiMo, can accept electrons in the presence of DCMU [20], and consequently is supposed to be insensitive to a modification of the Q_B site, and to measure the capacity of thylakoids to perform electron transfer up to Q_A [11]. To confirm this assumption, we compared oxygen evolution using SiMo as electron acceptor and Q_A photoreduction by changes of absorbance at 410 and 445 nm on thylakoids isolated from cells which have been photoinhibited for various lengths of time. Fig. 5 shows that there exists a very good correlation between the two types of measurement.

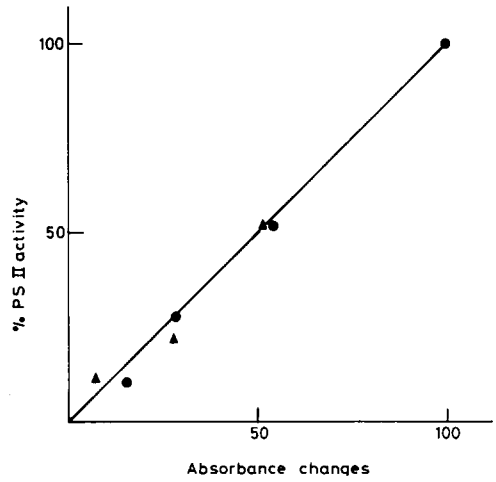

Fig. 5. Oxygen evolution using SiMo as electron acceptor versus flash induced absorbance changes at 410 nm (●) or 445 nm (▲). Thylakoids isolated from wild-type cells photoinhibited for different times were used for these measurements. 100% of PS II activity with SiMo: 85 μmol O_2/mg Chl per h.

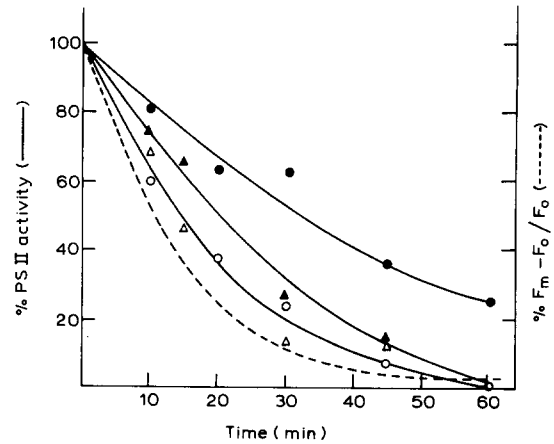

Fig. 6. PS II activity in wild-type (○, ●) and Az-V cells (△, ▲). Cells were incubated at high light intensity. At the times indicated, samples were taken for isolation of thylakoids. Oxygen evolution was measured using DCBQ (△, ○) and SiMo (▲, ●) as electron acceptors. 100% of PS II activity using DCBQ was 120 μmol O_2/mg Chl per h and that using SiMo was 95 μmol O_2/mg Chl per h. The decrease of F_v is also indicated in the graph (— — —).

Fig. 6 shows that during photoinhibition of wild type, the rate of decrease of oxygen evolution with DCBQ is faster ($t_{1/2} = 15$ min) than that with SiMo ($t_{1/2} = 35$ min). This figure also shows that in Az-V cells, the kinetics of decrease of oxygen evolution with DCBQ are the same as in the wild type ($t_{1/2} = 15$ min), whereas the decrease of oxygen evolution with SiMo is faster ($t_{1/2} = 20$ min) than that of the wild type.

Discussion

The behaviour of two mutants of *Synechocystis* 6714, DCMU-II_B and Az-V were compared to the wild-type during photoinhibition. Although their phenotypes are different, the mutant resistant to DCMU and the wild-type exhibited the same time-course of photoinhibition and of recovery in

dim light. In contrast, the mutant resistant to atrazine, Az-V, was unable to recover its PS II activity under low light illumination after being photoinhibited for 60 min; there was no synthesis and (or) integration of thylakoids proteins and the energy transfer from phycobilisomes to the membranes was decreased. Identical phenomena occurred in the wild type after a more prolonged exposure to high light illumination. It seems that the process of photoinhibition is composed of several steps and that Az-V cells reach an irreversible step faster than wild-type cells.

Radiolabeling experiments showed that under conditions of normal illumination the turnover of D_1 in mutant cells is identical to that of wild type i.e., their ability to synthesize and reincorporate D_1 is normal (Fig. 3). Therefore, mutant cells which were photoinhibited for only a short time (30 min), recovered similarly as wild type cells (Fig. 2B).

In Ref. 9 the authors correlate the loss of Q_A^- (or C-550) to the decrease of F_v during photoinhibition. They obtained a non-linear relationship which they suggested to be similar to that which holds between Q_A^- and F_v during the induction curve of fluorescence in the presence of DCMU [29]. We showed here (Fig. 6) that the decreases of F_v and electron flow through Q_B ($H_2O \rightarrow$ DCBQ) were similar and the fastest phenomena during photoinhibition. The loss of PS II electron transfer which does not involve Q_B ($H_2O \rightarrow$ SiMo) is slower than the former kinetics and it is different in wild-type and Az-V cells.

Fig. 7 shows the relationship between Q_A^- (measured as the complementary area over the induction curve) [23] and F_v during the induction curve of fluorescence in the presence of DCMU in the control samples (same curve for wild-type and Az-V). The activities are plotted versus F_v along photoinhibition on the same graph. The points corresponding to $H_2O \rightarrow$ DCBQ follow exactly the photoreduced Q_A^- curves and are similar for wild type and mutants. In contrast, SiMo activity measurements do no fall on the same curve and the deviation is different for wild type and Az-V; that difference gives a supplementary argument for the lack of correlation between the decrease of variable fluorescence and the damage to the centre itself (primary photochemistry).

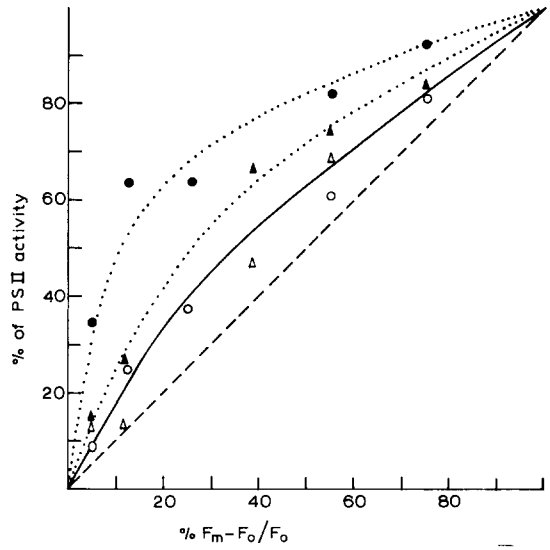

Fig. 7. Relationship between the loss of variable fluorescence and the decrease of PS II activity measured by oxygen evolution (·····) using DCBQ (△, ○) or SiMo (▲, ●) as electron acceptors in wild type (○, ●) and Az-V (△, ▲). The continuous line (———) represents the relationship between photoreduced Q_A^- (measured by the complementary area over the induction curve) and F_v during the induction curve of fluorescence induction in the presence of DCMU in control samples.

Therefore, we conclude that in our conditions of photoinhibition the decrease of F_v is related to the damage of the Q_B site which seems to be the primary target of photoinhibition. The reason for the loss of F_v remains obscure [11]. A more extensive inactivation of the RC II involving the decrease of oxygen evolution using SiMo (which parallels a decrease of Q_A photoreduction (Fig. 5)) occurs in a secondary step. This process occurs faster in Az-V than in the wild-type. This enhanced sensitivity of PS II to photoinhibition could be caused by a modification of D_1 in the mutant resistant to atrazine. This modification might accelerate damage to parts of the protein involved in other functions, e.g., in P-680 binding, or accelerate damage to D_2 and cytochrome *b*-559.

Since D_1 turnover is faster than D_2 turnover in normal photosynthesis conditions [24], it is thought that D_1 is particularly susceptible to damage by light (in the Q_B site?) and has to be frequently replaced by newly synthesized protein. It was proposed that the damaged protein is degraded by a protease [25]. The first cleavage site might be

localized in the loop region between helices IV and V [26] which is also involved in quinone and herbicide binding. It is possible that the signal for this degradation is a region (between Arg-225 and Arg-238) rich in glutamate, serine and threonine which resembles characteristic internal regions in eukaryotic proteins which are primary determinants for rapid degradation (PEST signal) [27].

In normal conditions, the continual and rapid replacement of D_1 allows an optimal functioning of PS II. However, during the photoinhibition, the modification of the Q_B site occurs so frequently that the repair process can no longer keep up with it. Perhaps the protease becomes unable to degrade all the damaged proteins. The excess of light on centers in disrepair increases the occurrence of more serious damage that results in complete inactivation of the RCII (D_1, D_2 and cytochrome *b*-559).

It is intriguing that the additional damage impedes repair. One possibility is that toxic agents which inhibit protein synthesis might be produced preventing the substitution of the degraded D_1. When protein synthesis stops, the process becomes irreversible.

The mutation which causes atrazine resistance in Az-V is supposed to be responsible for the increased sensitivity to high illumination. Sequencing of the *psbA* gene (coding for D_1 protein) isolated from Az-V mutant cells may help to clarify the role of D_1 in the process of photoinhibition. Such experiments are now in progress.

Acknowledgements

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