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Primary events occurring in photoinhibition in *Synechocystis* 6714 wild-type and an atrazine-resistant mutant

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Exposure of photosynthetic organisms to a light intensity higher than that needed to saturate photosynthesis causes the inhibition of Photosystem II activity (photoinhibition). We induced photoinhibition in cells of *Synechocystis* 6714 strains by exposure to light intensities between 1000 and 4000 $\mu E.m^{-2}.s^{-1}$. Fluorescence, thermoluminescence and oxygen measurements were used to follow the inhibition of electron transfer in Photosystem II centers. We demonstrated that, in oxygen evolving Photosystem II centers, the electron transfer from Q_A to Q_B was not slowed down. Photoinhibited samples presented a normal oscillation pattern of oxygen yield. The rate of deactivation of S_2 state was similar in control and photoinhibited cells. This indicates that the Q_A/Q_B equilibrium is not modified in the centers which evolve oxygen. By studying the kinetics of the decrease of the amplitudes of the thermoluminescence Q and B bands in *Synechocystis* 6714 we confirmed our previous conclusions drawn from electron transfer measurements: the inhibition of electron transfer to Q_B is faster than the inhibition of electron transfer to Q_A. We conclude that a succession of two different states of Photosystem II centers is produced during the process of photoinhibition: (1) A state in which the electron transfer is inhibited between Q_A and Q_B. (2) an inactivated low fluorescent state where Q_A^- formation is also inhibited. Restoration of the photoactive fluorescent Photosystem II requires de novo synthesis of D_1. Our main conclusion is that the first damage during photoinhibition in cyanobacteria cells is at the level of the Q_A to Q_B electron transfer step.

Introduction

Photoinhibition is related to the excess of light absorbed by the pigment antennae of the photosynthetic apparatus which cannot be properly dissipated by photosynthesis (reviewed in Refs. 1–4).

The target of photoinhibition is the Photosystem II (PS II). The process by which exposure to high light intensity results in a full inhibition of PS II centers and a total quenching of the variable fluorescence (F_v) remains to be elucidated. There is a general agreement that the first step of photoinhibition leads to an inhibition of electron transfer from H_2O to the plastoquinone pool and that D_1 degradation results from exposure to high light [1–4]. However, several contradictory hypotheses have been proposed. The contradictions seem to result partly from the type of biological material used and from different experimental conditions: light intensity, temperature, aerobiosis or anaerobiosis, etc... Research groups working with isolated thylakoids have produced some very clear results indicating that photoinhibition occurs between the P680 and Q_A [5–9]. Ohad et al. [10–12] working with *Chlamydomonas* cells suggested that the Q_B pocket of D_1 was the first site of damage. Our own experiments on the different behavior under photoinhibition of an atrazine-resistant mutant and the wild-type of *Synechocystis* 6714 also led us to invoke a first site of damage at the Q_B site [13]. In both strains, electron transfer through Q_B decreased more rapidly than the electron transfer which does not involve Q_B [10,13]. In living cells the repair process leading to reactivation of PS II activity involves D_1 synthesis and Photosystem II reassembly [10,13,14].

In the present work we have further characterized the cascade of events leading from an oxygen evolving PS II center with a large variable fluorescence to a low fluo-

Abbreviations: Chl, chlorophyll; D_1 and D_2, polypeptides of the RCII; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_0, F_v, F_{max}, initial variable and maximum fluorescence; P680, the chlorophyll molecule which acts as the primary electron donor in the RCII; PS II, Photosystem II; Q_A and Q_B primary and secondary quinone electron acceptors; RCII, reaction center II.

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rescent inactive PS II. Fluorescence, thermoluminescence and oxygen measurements were performed in wild-type *Synechocystis* 6714 and in the atrazine resistant mutant, AzV.

Materials and Methods

Growth conditions

Synechocystis 6714 cells were grown in the mineral medium described by Herdman et al. [15] with twice the concentration of nitrate and an illumination of about 70 $\mu E \cdot m^{-2} \cdot s^{-1}$. The cells were grown at 34° C. Other conditions were as previously described [16].

Photoinhibition experiments

Cells of *Synechocystis* 6714 wild-type or AzV mutant were harvested by centrifugation and resuspended in fresh growth medium at a final concentration of 30 μg Chl/ml. The cell suspension (15–30 ml) was incubated in a glass tube (3 cm diameter) refrigerated by cooled water and illuminated by one, two or four Atralux spots of 150 W (each giving an intensity of about 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$). The temperature of the different photoinhibitory treatments was as indicated in Results. The cells were gently stirred by a magnetic bar.

Fluorescence measurements

Fluorescence induction was determined with a fluorimeter described by Vernotte et al. [17]. The fluorescence was excited with a tungsten lamp through 5-59 and 4-96 Corning filters. The fluorescence was detected in the red region through a 2-64 Corning Filter and a Wratten 90 filter. The recording was done through a multichannel analyzer. The cell suspension contained about 1 μg Chl/ml. The F_{max} level was determined in the presence of 10^{-5} M DCMU.

The fluorescence decay after a short saturating flash was measured in an apparatus already described [18]. A pulsed light emitting diode (645 nm) was used as a non-actinic detecting beam (the pulses were 2 μs, spaced at 16 μs). The set of filters was a 4-96 Corning filter in front of the short saturating flash and a combination of a NV 550 (Schott), a RG 5 and an interference filter centered at 685 nm in front of the photomultiplier tube (S 20 light sensitivity). Under these conditions, less blue light falls on the RG 5 and the residual luminescence of the RG 5 filter is cut off by the interference filter.

The material (1.5 μg Chl/ml) was dark adapted for at least 10 min and the sample was renewed before each recording. To obtain a good signal-to-noise ratio, the curve was averaged 20 times.

Oxygen measurements

The amount of oxygen produced per flash during a sequence of saturating flashes was measured with a rate electrode equivalent to that described by Joliot and Joliot [19]. The short (5 μs) saturating flashes were produced by a Strobotac (General Radio Company). The spacing between flashes was 0.6 s. Each experiment was started with dark-adapted cells (500 μg Chl/ml).

Thermoluminescence

The thermoluminescence cuvette, 1 mm thick, was formed by a rubber plate with a 1×2 cm cavity, pressed between a Plexiglass window and an aluminium plate which could be dipped partly or totally in liquid nitrogen. A 'Thermocoax' heater, on the other side of the plate, was used for regulation of temperature which was measured in the cuvette by a thermocouple. Samples were prepared just before each measurement, by centrifugation of cell cultures and resuspension at 400 μg chlorophyll/ml (in the presence of 25% glycerol).

The photoinhibitory illumination produces a modification in the charge distribution on the electron transport chain between the two photosystems as compared to the control. For B band measurements, the samples were preilluminated by a sequence of 20 flashes in order to homogenize the charge distribution. Then, after 5 min of dark incubation, a flash was given at 2° C and the sample was rapidly frozen. For the detection of the Q band, DCMU was added after dark adaptation, and the flash was given at −20° C.

After 30 s of temperature equilibration at −40° C, the temperature was linearly increased to +80° C in 4 min (0.5 C°/s). The luminescence emission was measured, at wavelengths above 650 nm, by a cooled photomultiplier connected to a photon counting system. Signal recording and temperature regulation were performed by a PC compatible microcomputer. The signal was treated by a Fourier transform program.

Results

Parameters influencing photoinhibition

Photoinhibition in vivo is influenced by different parameters such as light intensity, temperature and protein synthesis. Recovery from photoinhibition involves de novo synthesis of thylakoid proteins among which the most prominent is D_1. In living cells, the repair process occurs concomitantly with photoinhibition.

During photoinhibition of cyanobacteria cells, the F_v decrease is due to a quenching of F_{max}. F_0 remains constant. We have already shown that the decreases of F_v and of electron flow through Q_B ($H_2O \rightarrow$ DCBQ) are closely related [13].

We have studied the effect of protein synthesis on the decrease of the variable fluorescence (F_v) in *Synechocystis* 6714. Fig. 1A shows the F_v decrease in cells exposed to a light intensity of 1000 $\mu E \cdot m^{-2} \cdot s$ at 34° C which is the optimal temperature for *Synechocystis* growth. F_v decreased more rapidly in cells in which

protein synthesis has been inhibited by chloramphenicol. When light intensity was greater, the effect of the repair process was less marked (Fig. 1A). Protein synthesis could no longer keep up with protein damage when light intensity was increased and the temperature was lowered at the same time. Chloramphenicol addition did not modify the kinetics of photoinhibition at 25 °C with a light intensity of 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 1B). Therefore, all the following experiments described in this work were performed at 25 °C in order to avoid recovery effects due to protein synthesis.

When photoinhibited wild-type cells were transferred to low light (or dark) in the presence of chloramphenicol, a small recovery of F_v (10%) was observed. Fig. 2 shows the increase of F_v during recovery of wild-type and AzV-photoinhibited cells at low light in the presence or absence of chloramphenicol. Total recovery of PS II activity was obtained in both strains in the absence of chloramphenicol. A small recovery of F_v was observed in the presence of chloramphenicol only in wild-type cells at room temperature. At 4 °C, this partial recovery did not occur. The partial recovery might be related to conformational changes of the D_1 protein hindered by low temperature or by the mutations in the Q_B pocket in the AzV mutant.

The influence of light intensity and temperature is dealt in detail in another publication [20]; however, we want to stress here the most important points. Fig. 3 shows the decrease of F_v during photoinhibition under differents light intensities at 5 °C (Fig. 3A) and at 25 °C (Fig. 3B). F_v was affected only by F_{max}, since F_0 remained constant under all the conditions. At any given temperature the decay of variable fluorescence was accelerated by increasing light intensity and at any given light intensity photoinhibition occurred more slowly at 5 °C than at 25 °C. PS II activity estimated from oxygen yield under continuous illumination decreased also more slowly at 5 °C than at 25 °C [20]. Surprisingly, the low temperature did not increase photoinhibition in *Synechocystis* as described for higher plants (reviewed by Oquist et al. [21]); on the contrary, it had a protective effect from high light.

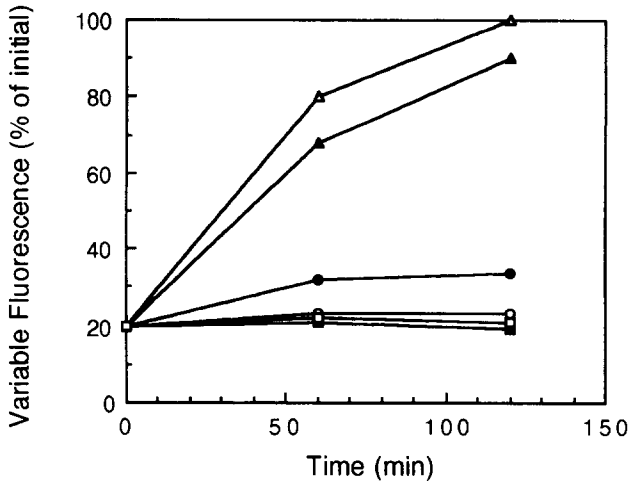

Fig. 2. Partial recovery of the variable fluorescence in the presence of chloramphenicol. Wild-type (closed symbols) and AzV (open symbols) cells were exposed to a light intensity of 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 25 °C in the presence of chloramphenicol. Samples were washed nd transferred to low light in the absence of chloramphenicol at 34 °C (triangles) or in the presence of chloramphenicol at 34 °C (circles) or at 4 °C (squares). The partial recovery of F_v observed in the presence of chloramphenicol, also occurred in the dark.

Oxygen evolution

The amount of oxygen evolving PS II centers can be deduced from the average oxygen yield per flash during a train of short saturating flashes.

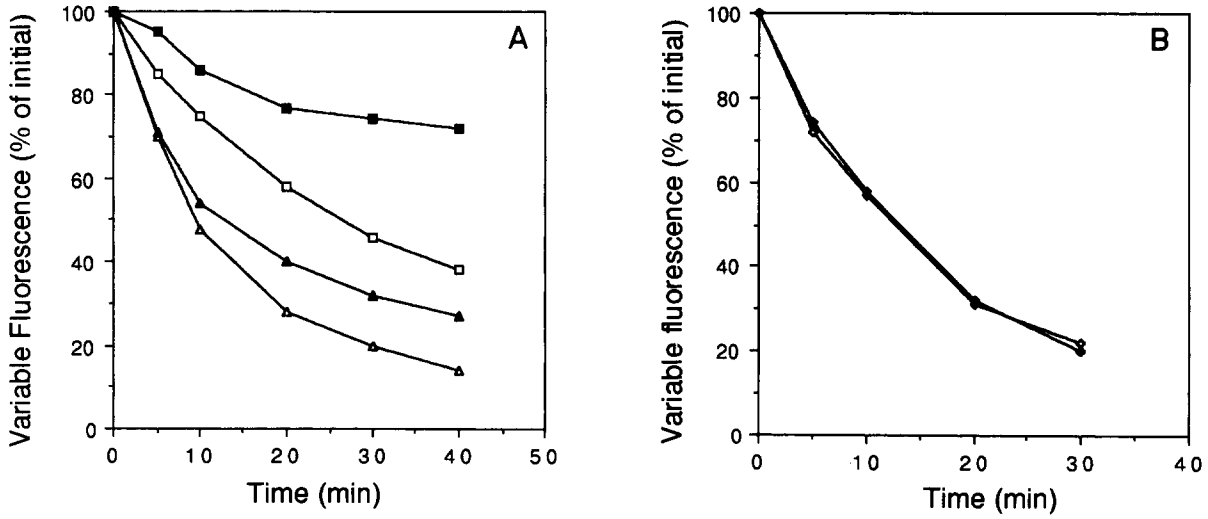

Fig. 1. Effect of protein synthesis on the decrease of variable fluorescence during photoinhibition. (A) *Synechocystis* cells were exposed to a light intensity of 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (□–□) or 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (△–△) at 34 °C, in the presence (open symbols) or in the absence (closed symbols) of chloramphenicol. (B) *Synechocystis* cells were exposed to a light intensity of 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 25 °C in the presence (open symbols) or in the absence (closed symbols) of chloramphenicol. $F_v/F_0 = 1.5$ in cells non-photoinhibited.

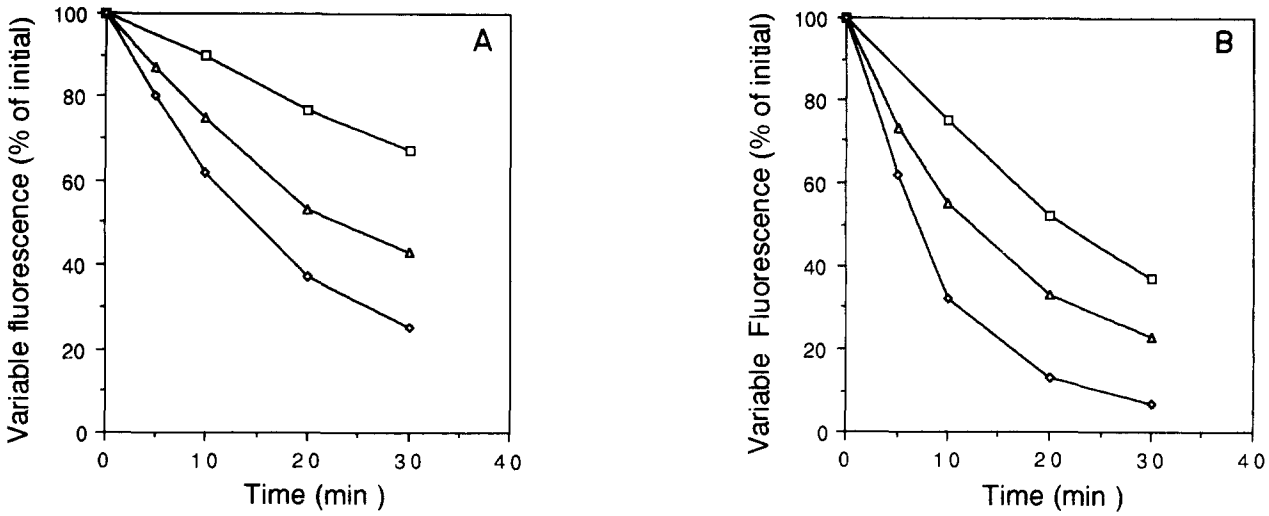

Fig. 3. Effect of light intensity and temperature on the decrease of variable fluorescence during photoinhibition. The cells were exposed to the light given by one (□), two (△–△) or four (◇) spot lamps. Each lamps gived an intensity of 1000 $\mu E \cdot m^{-2} \cdot s^{-2}$. (A) Photoinhibition at 5 °C. (B) Photoinhibition at 25 °C.

Fig. 4 shows four oxygen sequences corresponding to samples photoinhibited for 4, 8 or 15 min at 25 °C with four lamps and to a control sample. There was a marked decrease in the amount of active centers but the oxygen oscillations were strictly superimposable when the sequences were normalized to the yield of the third flash. The deactivation kinetics of S_2 were also identical in the four samples ($t_{1/2}$ = 20 s at 20 °C). These results showed that during photoinhibition no modified state of oxygen-evolving PS II centers was detected. The concentration of active centers diminished with the duration of photoinhibition. The centers which no longer emitted oxygen were in two different states as shown by fluorescence measurements.

Fluorescence experiments

We have previously observed that electron transfer to Q_B was inhibited prior to electron transfer to Q_A during photoinhibition in the wild-type and the Az-mutant of *Synechocystis* 6714 [13]. We also showed that the rate of decrease of oxygen evolution measured with the external acceptor SiMo was faster in the mutant than in the wild-type [13].

We wanted to know whether, as expected from the former results, there were some centers inactive in oxygen evolution but still fluorescent where Q_A^- was still formed. The fluorescence decay, after a saturating flash, monitors the reoxidation of Q_A^- by Q_B and the distribution of electron between Q_A and Q_B after stabilization. If some centers are blocked between Q_A and Q_B and if they are fluorescent they will contribute to the slow phase of the fluorescence decay.

The total amplitude of the variable fluorescence detected after the flash decreased with increasing duration of photoinhibition, in accordance to observations under continuous illumination (data not shown).

The initial slope of the decay, a parameter which reflects the Q_A^- to Q_B electron transfer rate, was not modified in photoinhibited cells. On the other hand, the amount of slow phase was larger in the photoinhibited than in the control sample (Fig. 5). The increase in the amount of slow phase with the duration of photoinhibition was larger in wild-type cells as compared to the mutant cells (Fig. 5). Thus, as it was seen with partial electron transfer measurements [13], the formation of Q_A^- was inhibited faster in AzV cells than in wild-type cells. The amplitude of fluorescence is not linearly related to the amount of Q_A^- and other parameters can influence the slow phase amplitude. However, qualitatively, our results show that there is an increase in the

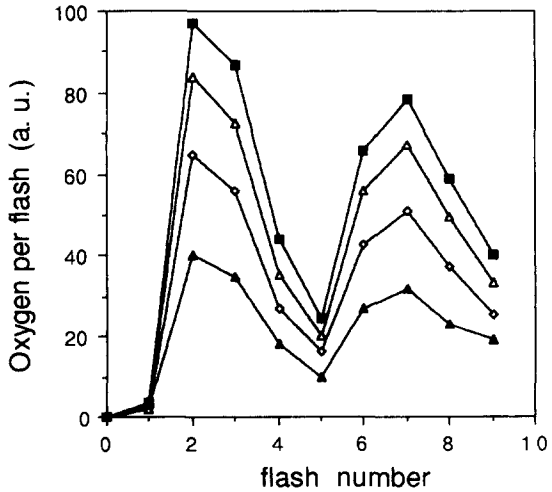

Fig. 4. Oxygen yield per flash during a series of short saturating flashes in control (■–■) and photoinhibited cells for 4 min (△), 8 min (◇) and 15 min (▲) at 25 °C with 4 lamps. When the amplitudes were normalized to the amount of oxygen evolved in the third flash, the sequences were identical.

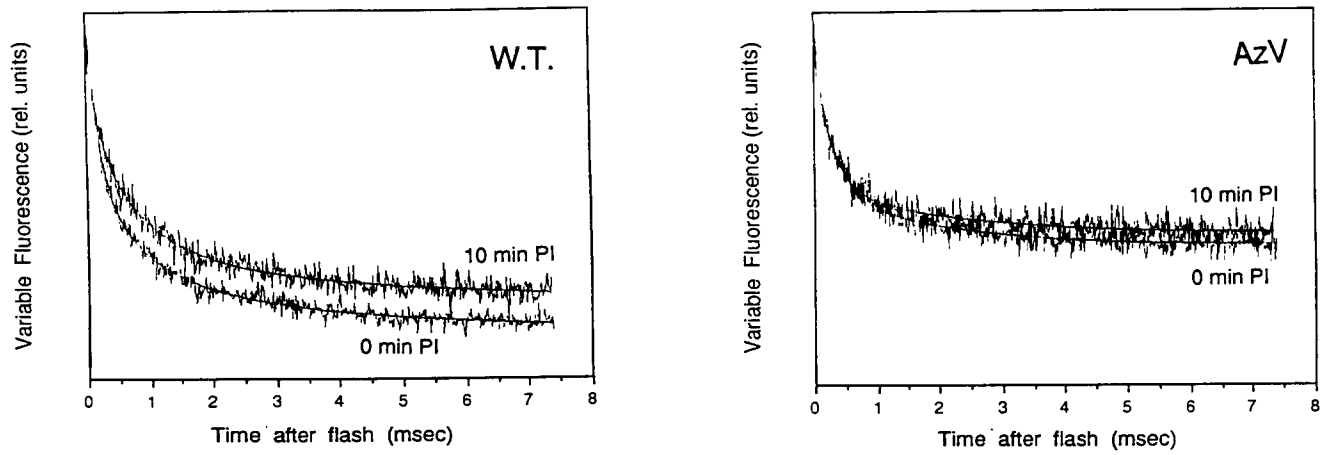

Fig. 5. Fluorescence decay after one saturating flash in dark adapted material. The lower recording corresponds to the control, the upper recording corresponds to a sample photoinhibited for 10 min with two lamps at 25 °C. The fluorescence of wild-type (W.T.) and Az mutant was recorded between 120 μs and 7.5 ms. F_{max} have been computed from curve fitting and normalized.

amount of fluorescent centers in which Q_A^- is reoxidized only by the slow back-reaction with the S_2 state. Their concentration increases with increasing duration of the exposure to high light.

Thermoluminescence measurements

Charge recombination gives rise to thermoluminescence which enables the detection and quantitation of the $S_2Q_A^-$ (Q band) and $S_2Q_B^-$ (B band) states and of their stability during photoinhibition [22]. In *Chlamydomonas reinhardtii* Ohad et al. [11,12] have observed a large shift of the B band towards low temperature during photoinhibition; the authors attributed the shift to a reversible modification of the Q_B site. In isolated chloroplasts Vass et al. [8] have clearly shown that the peak positions of the B and Q thermoluminescence bands were not affected by photoinhibition; in addition, B and Q bands declined in parallel during the photoinhibitory illumination.

In *Synechocystis* we found another type of behavior. Fig. 6 shows the B band in AzV and wild-type photoinhibited cells compared to control cells. The difference in the peak position of the B band between AzV and wild-type cells (respectively 32 and 38 °C) is due to a change in the equilibrium between Q_A and Q_B in the mutant as compared to the wild-type [23]. The B band was not shifted towards lower temperature during photoinhibition in the wild-type but it was slightly displaced (4–5 C°) in AzV (Fig. 6). In both strains the decay of the Q band was slower than the decay of the B band indicating that, during photoinhibition, electron

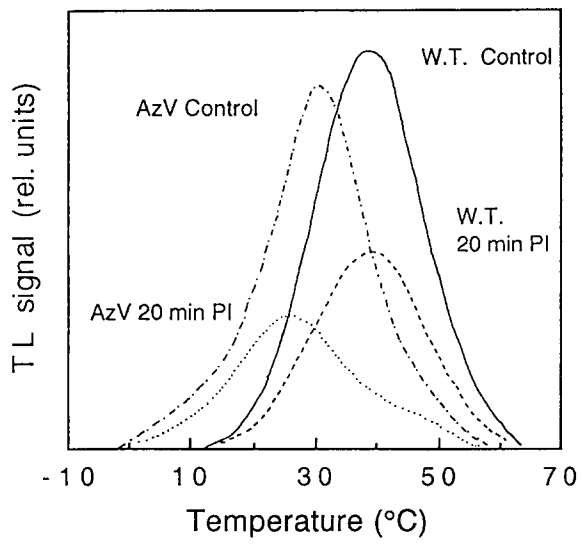

Fig. 6. The B band of thermoluminescence in wild-type (———) and AzV (–·–·–)control cells and in wild-type (- - -) and AzV (·····) photoinhibited cells for 20 min at 25 °C with a light intensity of 2000 $\mu E \cdot m^2 \cdot s$. The sample were preilluminated by a sequence of 20 flashes before dark adaptation, in order to homogenize the charge distribution in the electron transfer chain between the two photosystems.

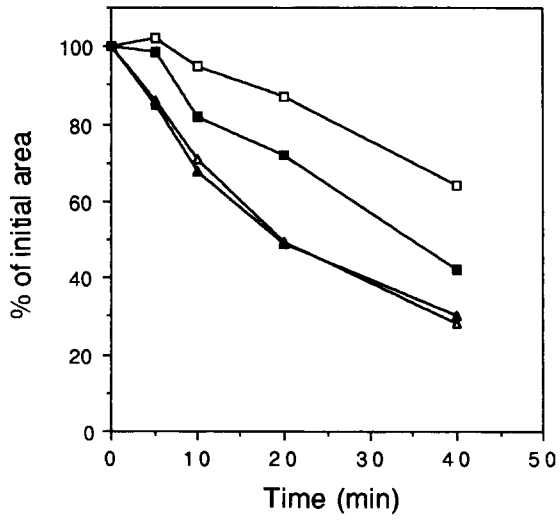

Fig. 7. Decrease in the areas of the B band (triangles) and the Q band (squares) as a function of photoinhibition duration of wild-type cells (open symbols) and AzV cells (closed symbols).

transfer inhibition from H_2O to Q_B occurred prior to the inhibition of electron transfer from H_2O to Q_A (Fig. 7). The decrease in the B band was similar in both strains (Fig. 7). However, the decay of the Q band was faster in AzV than in the wild-type, confirming that the inhibition of Q_A photoreduction is accelerated in AzV (Fig. 7).

Discussion

Photoinhibition in *Synechocystis* 6714 leads to the inhibition of PS II activity as in all other oxygen-evolving systems studied at the present time. We have already shown that the disruption of the electron transfer from Q_A to Q_B was the first step of the process leading to the total inactivation of PS II [13].

In the present report we have studied in more detail the electron transfer during photoinhibition and the different states of the centers during this process. Some PS II centers remained for some time in an oxygen-evolving normal state with no detectable change in their organization. The concentration of this state decayed rather slowly when compared to the average number of photons falling on PS II centers. A modification at the level of the Q_B site produced subsequently an inhibition of the electron transfer from Q_A to Q_B. The centers were still able to perform a charge separation and Q_A photoreduction. Shortly after, further damage occurred and it produced a low fluorescent photoinactive state of the PS II center. If the exposure time to high light was too long, the cell lost the ability to replace the damaged protein and the process became totally irreversible, leading to cell death.

The only genotypic difference between the AzV mutant and the wild-type resides in two point mutations (Ala-251 → Val and Phe-211 → Ser) located in the Q_B pocket domain of D_1 [24]. We have demonstrated that these mutations were sufficient to produce an increased sensitivity of AzV to high light and an accelerated cell death. We have also observed that in AzV the inhibition of electron transfer from H_2O to Q_A was faster than in the wild-type [13]. In this study we confirmed that in AzV cells the process leading to the inhibition of Q_A photoreduction was accelerated. This was shown by the faster decay of the thermoluminescence Q band and the smaller increase in the amount of slow phase in the fluorescence decay kinetics.

In *Chlamydomonas reinhardtii*, we have observed a transient formation of a high fluorescent state giving rise to an appreciable increase of the initial fluorescence level [20]. Ohad et al. [11,12] also described some reversible steps during photoinhibition. Although we worked with living cells of an unicellular organism, we did not detect in *Synechocystis* any large reversible effect of photoinhibition. However, some of our results suggested that small reversible conformational changes of the PS II centers might occur before the irreversible inhibition of the electron transfer to Q_B in *Synechocystis*. Photoinhibited wild-type cells, but not AzV cells, recovered 10% of their variable fluorescence in the dark and in the presence of chloramphenicol. A slight shift of the B band of thermoluminescence was observed in photoinhibited AzV cells but not in wild-type cells. These differences between the behavior of the wild-type and the mutant might be connected to the difference in the structure of the D_1 protein.

A variety of different factors have been suggested as the direct cause for photodamage, depending on the biological material or the experimental conditions used. We think that a more realistic view should consider that photoinhibition is the consequence of the effect of all the multiple factors on the PS II center. At any given temperature (from 5°C to 34°C), PS II activity decreased more rapidly when light intensity was increased, indicating that the frequency of the photons falling on PS II is one of the parameters involved in this process. Absorption of the excess of light energy by the pigments might lead to the formation of toxic radicals. The rate of electron hole pair formation at the reaction center is much faster than oxygen evolution or plastoquinone reduction. This leads to the accumulation of oxidized *S* states, tyrosine radical and reduced electron acceptors. Lowering the temperature or increasing the light intensity enhances the ratio of the frequency of charge separation versus the frequency of positive and negative charge evacuation from PS II. Therefore, if PS II damage was strictly governed by the accumulation of strong oxidants and reductants, increasing light or lowering temperature should have the same effect on the extent of photoinhibition. We showed that the rate of photoinhibition was slowed down when the temperature was lowered, at a given light intensity. On the other hand, under anaerobic conditions, photoinhibition also takes place [7,25,26] and leads to inactivation of PS II. Therefore, neither the oxygen radicals nor the accumulation of strong oxidants and reductants can be taken as the unique cause of PS II damage. Since we have shown elsewhere that the state of the membrane lipids has a clear influence on the rate of photoinhibition [20]. we think that conformational changes of the PS II proteins (mostly D_1 but possibly D_2) and proteolytic activities are also important factors involved in photoinhibition.

Experiments are still needed to fully understand the complexity of photoinhibition, but we have clearly demonstrated that in *Synechocystis* 6714, each PS II can be found (as far as electron flow is concerned) in three different states during photoinhibition: unmodified active centers, centers in which the electron transfer between Q_A to Q_B was inhibited, and fully inactivated low fluorescent centers.

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