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Photoinhibition: Impairment of the primary charge separation between P-680 and pheophytin in photosystem II of chloroplasts

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The question of the primary site of photoinhibition was investigated using the light-induced absorbance change of pheophytin *a* (at 685 nm) in spinach chloroplasts and *Chlamydomonas reinhardtii* cells. Photoinhibition of spinach thylakoid membranes resulted in a parallel decrease in the amplitude of the pheophytin *a* (ΔA_{685}) and Q_A (ΔA_{320}) photoreduction signals. In intact *Chlamydomonas reinhardtii* cells the pheophytin photoreduction and oxygen evolution activity exhibited a similar decrease during photoinhibition. A complete recovery of both activities was attained within 60 min incubation in normal growth conditions. It is concluded that the primary site of photoinhibition involves the components (P-680 and/or pheophytin) of the primary charge separation.

Photoinhibition; Charge separation; Pheophytin; Electron transport; (Spinach chloroplast, *Chlamydomonas reinhardtii*)

1. INTRODUCTION

Photoinhibition was originally described by Kok [1] as a decrease in the rate of photosynthesis of green algae when exposed to high light intensities. The adverse response to high light was also observed in higher plants. Fluorescence and/or electron transport measurements indicated that the site of high-light-induced damage is located at or near PS II [2]. The molecular mechanism of photoinhibition has attracted considerable attention recently. In this respect, two schools of thought prevail. One [3] postulates that photoinhibition results from a light-dependent damage to the plastoquinone binding site of PS II (Q_B-binding protein). Loss of function of the Q_B-binding site in the 32 kDa apoprotein of PS II would lower the rate of PS II electron flow and, therefore, overall photosynthesis. According to this model, photoinhibition does not entail damage to the donor side or to the reaction center of PS II [3]. The attractive aspect of this model is the connection of photoinhibition with the rapid turnover of the 32 kDa Q_B-binding protein, a phenomenon observed under physiological conditions [4].

A large group of investigators, however, advocate a primary site of photoinhibition closer to the photochemical reaction center of PS II [2,5–7]. Recent work from this laboratory has shown that photoinhibition of photosynthesis is manifested by a lowered amplitude of the photoreduction of the primary quinone acceptor Q_A of PS II [7]. Until now, this measurement has been the most direct and localized test on the primary site of photoinhibition because it probed the function of the charge separation between

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Abbreviations: Chl, chlorophyll; PS, photosystem; Q_A, primary quinone acceptor of PS II; Pheo, pheophytin primary electron acceptor of PS II; P-680, photochemical reaction center of PS II; C_f, flattening correction factor

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P-680, pheophytin and Q_A in the PS II reaction center. The PS II charge separation reaction is summarized as follows:

$$P680^* \cdot Pheo \cdot Q_A \longrightarrow P680^+ \cdot Pheo^- \cdot Q_A \longrightarrow P680^+ \cdot Pheo \cdot Q_A^-$$

Since photoinhibition adversely affected the formation of semiquinone anion (Q_A^-), it was concluded that the mechanism of photoinhibition involves a direct photochemical reaction center inactivation [7] and that any photoinhibition-related effects on the reducing side of PS II [3] must represent secondary phenomena.

However, in terms of the charge separation reaction shown above, it is still unclear whether photoinhibition adversely affects the primary charge separation reaction between P-680 and Pheo (step 1) or the transfer of the reducing equivalent from $Pheo^-$ to Q_A (step 2). In the present work we monitored the primary charge separation reaction of PS II by measuring the photoreduction of Pheo in isolated thylakoid membranes and in intact cells of *Chlamydomonas*, following the methodology developed by Klimov et al. [8]. Our results show that the primary charge separation between P-680 and Pheo is adversely affected during photoinhibition.

2. MATERIALS AND METHODS

Spinach chloroplasts and PS II enriched particles (BBY [9]) were isolated [10]. *Chlamydomonas reinhardtii* cells were grown and harvested [11]. Photoinhibition treatments of chloroplast and *Chlamydomonas reinhardtii* cell suspensions were performed [7]. Light-induced absorbance change measurements of Pheo *a* were taken in an anaerobic cell of 1 cm pathlength as described [8] with a laboratory constructed split beam spectrophotometer [12]. In order to alleviate light scattering and sedimentation of thylakoid membranes in the cuvette, 0.005% (v/v) Triton X-100 was added to the samples prior to the measurement. The Pheo photoreduction was induced by blue (Corning CS 4-96) excitation light at an intensity of $600\,\mu E \cdot m^{-2} \cdot s^{-1}$ ($\sim 1.2 \times 10^5$ $erg \cdot cm^{-2} \cdot s^{-1}$). The half-bandwidth of the measuring beam was 1 nm. The absorbance difference measurements were corrected for the effect of particle flattening [13]. Whole chain oxygen evolution of the *Chlamydomonas reinhardtii* cells ($H_2O \longrightarrow CO_2$) was assayed at saturating light intensity using a Clark type oxygen electrode [11].

3. RESULTS AND DISCUSSION

Fig.1. shows the wavelength dependence of the light-induced absorbance change obtained with resolved membranes of the grana partition regions suspended under reducing conditions (E_h = −490 mV). The difference spectrum shows minor positive bands at 658 and 675 nm and a major negative band at 685 nm. The position of the peaks and the narrow band width of the 685 nm band

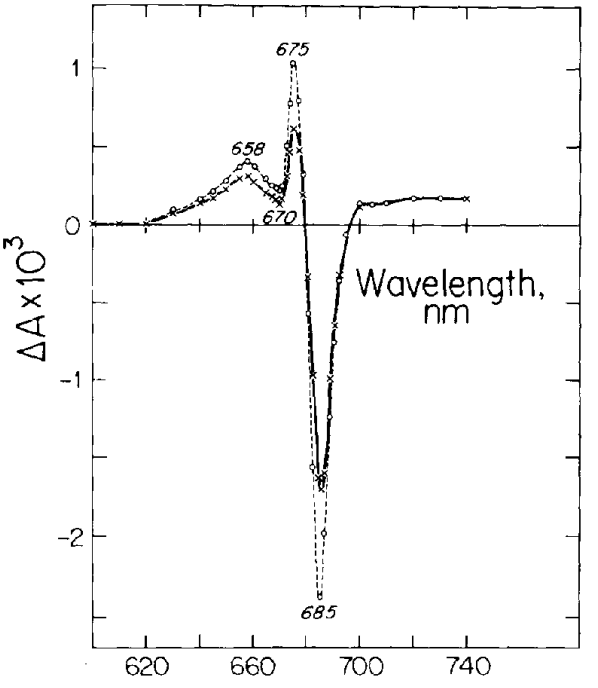

Fig.1. Light-induced absorbance difference spectrum of thylakoid membranes from the grana partition regions (BBY particles) of spinach chloroplasts suspended at a redox potential of −490 mV (×). The reaction mixture contained 20 mM Tris-HCl (pH 7.8), 35 mM NaCl, 2 mM $MgCl_2$, 2 μM methylviologen, 2 μM indigodisulphonate, 2 mM $MnCl_2$ and 0.005% (v/v) Triton X-100. The excitation light intensity was $600\,\mu E \cdot m^{-2} \cdot s^{-1}$ ($\sim 1.2 \times 10^5$ $erg \cdot cm^{-2} \cdot s^{-1}$). Optical pathlength of the cuvette was 1 cm and the Chl concentration was 7.8 μM. The same spectrum corrected for particle flattening (○).

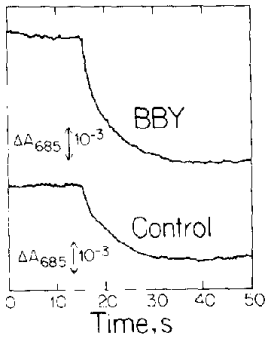

Fig.2. Time-course of Pheo photoreduction as indicated by the bleaching at 685 nm. The reaction mixture contained resolved membranes from the grana partition regions (BBY) or unfractionated thylakoid membranes (control) of spinach chloroplasts. The actinic light came on at about 15 s. Chl ($a+b$) concentration was 17 μM. Differential flattening correction factors at 685 nm were 1.29 (BBY) and 1.26 (Control).

identify the difference spectrum as arising from the photochemical reduction of the Pheo primary electron acceptor of PS II [8]. The same spectrum corrected for particle flattening [13] is shown in fig.1, dashed line.

Fig.2 shows typical traces of the light-induced absorbance change at 685 nm (ΔA_{685}) obtained either with resolved membranes from the grana partition regions (BBY) or with isolated but intact thylakoid membranes (control) poised at a redox potential of about -490 mV. Being on an equal Chl basis, the amplitude of the signal obtained with the membranes of the grana partition regions (BBY) is considerably greater than that of the unfractionated thylakoids (control), suggesting Pheo enrichment in the BBY particles. The quantitation of Pheo a from the ΔA_{685} must be based on a differential extinction coefficient, for which published values range from 32 to 100 $mM^{-1} \cdot cm^{-1}$ [14–16]. We estimated the differential extinction coefficient of Pheo a in BBY particles directly by using the known $Chl/Q_A = 230:1$ in BBY particles [10,17] and by assuming a 1:1 ratio for $Pheo/Q_A$ photoreduction. An absorbance change of $\Delta A_{685} = -3.72 \times 10^{-3}$ in BBY particles (fig.2, upper), after correction for the effect of flattening ($C_f = 1.29$), implies an in situ differential extinction coefficient of 65 $mM^{-1} \cdot cm^{-1}$, in good agreement with the $\Delta\epsilon$ of P-700 and P-680 [18,19]. The in vivo concentration of Pheo a in unfractionated spinach thylakoids was then estimated from the $\Delta A_{685} = -2.27 \times 10^{-3}$ (fig.2, lower) after correction for particle flattening ($C_f = 1.26$). A Chl/Pheo = 386:1 was derived, in good agreement with the reported PS II concentration ($Chl/Q_A = 380:1$) in unfractionated spinach chloroplasts [10]. It is implied that 1 Pheo molecule per PS II complex can be photoreduced upon poising the oxidation-reduction potential of the chloroplast suspension medium to a negative (-490 mV) value.

Having established the conditions for the measurement of Pheo photoreduction in spinach chloroplasts, we proceeded with our photoinhibition treatment [7]. Fig.3 compares the amplitude of the light-induced ΔA_{685} in dark-adapted (control) and thylakoid membranes subjected to 60 min photoinhibition treatment. It is evident that the amount of photoreducible Pheo is lowered upon photoinhibition. Such a response is reminiscent of the lowered amount of photoreducible Q_A occurring upon photoinhibition [7]. Fig.4 compares the time course of the amplitude decrease of ΔA_{320} (Q_A photoreduction) and of ΔA_{685} (Pheo photoreduction) during photoinhibition. It is evident that the two phenomena display identical kinetics, suggesting that the reduction of Pheo is adversely affected and that inhibition in Q_A photoreduction [7] is only a consequence of inhibition in the primary charge separation. It is unequivocally concluded that during photoinhibition

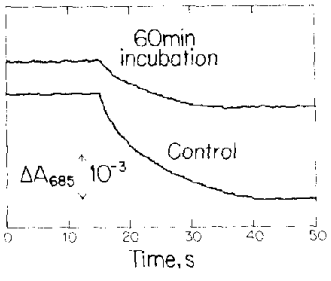

Fig.3. The effect of photoinhibition on the Pheo photoreduction in spinach thylakoid membranes. The light-induced absorbance change was measured at 685 nm at a redox potential of -490 mV. The concentration of Chl ($a+b$) was 17 μM. Control thylakoids were kept in the dark and photoinhibited thylakoids were incubated for 60 min at 0°C under strong white light (2500 $\mu E \cdot m^{-2} \cdot s^{-1}$) prior to the measurement. Note the substantially lower amplitude of Pheo photoreduction (ΔA_{685}) upon photoinhibition.

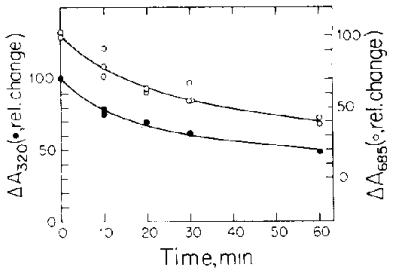

Fig.4. The amplitude of the light-induced absorbance change reflecting Q_A photoreduction (ΔA_{320}, •) and Pheo photoreduction (ΔA_{685}, ○) as a function of incubation time under strong actinic illumination (photoinhibition). Note the identical Q_A and Pheo dependence on photoinhibition.

Table 1

Photoinhibition and recovery parameters in *Chlamydomonas reinhardtii*

Treatment	Rate of oxygen evolution, % of control	ΔA_{685} % of control
Control	100	100
60 min photoinhibition	63	45
+ 60 min recovery	120	119

The light-saturated rate of oxygen evolution (P_{max}) of the control was 173 μM $O_2 \cdot \mu$M Chl$(a+b)^{-1} \cdot h^{-1}$ and the Chl/Pheo ratio was 510 ± 50

the primary charge separation between P-680 and Pheo in PS II of chloroplast is prevented.

Our conclusion on the primary site of photoinhibition is consistent with the results of several other investigators. Thylakoid membranes with inactivated water-splitting enzyme exhibited accelerated rates of photoinhibition [20] suggesting a primary site between Z and Q_A [21] or Z and P-680 [22,23]. Additional work with chloroplasts has suggested that photoinhibition is not specific to the Q_B-binding site [5,6]. Moreover, our results exclude the secondary donor Z as the primary site of photoinhibition because under our experimental conditions (in the presence of dithionite, E_h = −490 mV), the measurement of Pheo does not require an electron transfer reaction between Z and P-680. However, conclusions on the primary site of photoinhibition resulting from this and other work [2,5–7,20–23] is in contrast to the model advocating the Q_B-binding site as the locus of photoinhibition [3,24].

Measurements of photoinhibition with the Pheo photoreduction assay were extended to include the green alga *Chlamydomonas reinhardtii*. Cells grown at a medium light intensity (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) displayed Chl/Pheo = 510:1 ratios, similar to Chl/Q_A = 490:1 reported previously [11]. Photoinhibition was administered by a high-light treatment (3000 $\mu E \cdot m^{-2} \cdot s^{-1}$) to continuously stirred and bubbled (3% CO_2 in air) cultures at 25°C. Prior to the Pheo quantitation measurement, *C. reinhardtii* cells were sonicated briefly (1 min in pulsed mode) in order to disrupt the cell wall and facilitate the equilibration of the thylakoid membrane with the redox mediators at the −490 mV redox potential of the medium. Photoinhibited cells manifested a decrease in light saturated rates of O_2 evolution (P_{max}, measured under in vivo conditions) and a similar decrease in the light-induced signal of Pheo photoreduction (table 1). Moreover, recovery of P_{max} after returning the cells to normal growth conditions was paralleled by recovery of the Pheo photoreduction signal. It is concluded that both photoinhibition and the physiological process of chloroplast recovery from it involve the function of the primary photochemical charge separation between P-680 and Pheo in the reaction center of PS II. Hence, our results do not support the notion of a primary effect of photoinhibition on the Q_B-herbicide binding site of the 32 kDa protein [3]. Instead, it appears that photoinhibitory damage occurs at the photochemical reaction center of PS II, i.e., close to, if not at, P-680.

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