Thermoluminescence Investigation of Photoinhibition in the Green Alga, *Chlamydobotrys stellata* and in *Pisum sativum* L. Leaves

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During photoinhibitory light treatment of the green alga, *Chlamydobotrys stellata* and *Pisum sativum* leaves the amplitude of the B thermoluminescence band (S\(_2\) Q\(_A^+\) charge recombination) decreased faster than that of the Q band (S\(_2\) Q\(_B^-\) charge recombination). Consistently, in the alga cells the electron transport rate from water to oxidized diaminodurene (electron acceptor after Q\(_B^+\)) was also diminished faster than that measured from water to phenyl-\(p\)-benzoquinone (electron acceptor after Q\(_A^-\)). These observations suggest that in intact photosynthetizing organisms at high light intensities on the acceptor side of photosystem II photo-inhibition is initiated at the binding site of the secondary quinone acceptor, Q\(_B^-\).

Introduction

Exposure of photosynthetizing organisms to higher light intensities than that which can be utilized in photosynthesis or dissipated in harmless processes results in a loss of photosynthetic activities [1]. The phenomenon is called photoinhibition. Photoinhibition is manifested as an inhibition of photosystem II (PS II) electron transport which is followed by a degradation of the 32 kDa Q\(_B^-\) binding D1 protein of the PS II multiprotein complex [1, 2].

Different components of the PS II reaction center were proposed to be the primary action site of photoinhibition. It was suggested that at low light intensities the inhibition of electron transport and degradation of the D1 protein is triggered at the donor side of PS II by accumulation of tyrosine Z\(^+\) and/or P680\(^+\), both of which are highly oxidizing [3–5]. At high light intensities photoinhibition is due to overreduction of the acceptor side of PS II [4, 6]. In isolated chloroplasts or thylakoids the P680-Phe-Q\(_A^-\) section of electron transport chain proved to be the primary target of photoinhibition [6–10]. However, in intact algae cells the Q\(_B^-\) binding site located within a hydrophobic pocket of the D1 protein [11] was suggested to be the first site of photoinhibition [2, 12–15]. A communication between the Q\(_B^-\) and Q\(_A^-\) binding sites via a recently proposed new contact site [16] between the D1 and D2 proteins may facilitate the propagation of the photoinhibitory process.

In order to determine the in vivo primary action site of photoinhibition we carried out a detailed thermoluminescence (TL) investigation of the green alga, *Chlamydobotrys stellata* and pea leaves during photoinhibitory light treatment. The thermoluminescence light emission of photosynthetic organisms is associated with charge recombination occurring between positively charged donors and negatively charged acceptors of photosystem II [17]. The two main TL bands appearing at around +10 and +30 °C are designated as Q and B bands, respectively. In the present work it was found that during photoinhibition the B thermoluminescence band (S\(_2\) Q\(_B^-\) charge recombination) diminished faster than the Q band (S\(_2\) Q\(_A^-\) charge recombination) suggesting that in vivo at high light intensities

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**Abbreviations:** Phe, pheophytin; PS II, photosystem II; P680, reaction center chlorophyll of PS II; Q\(_A^-\), primary quinone acceptor of PS II; Q\(_B^-\), secondary quinone acceptor of PS II; Q\(_B^+\), primary negatively charged acceptor of photosystem II; Q\(_A^-\), secondary negatively charged acceptor of photosystem II; TL band associated with S\(_2\) Q\(_A^-\) charge recombination; B band, TL band associated with S\(_2\) Q\(_B^-\) charge recombination; S\(_2\), oxidation state of the water-splitting system; TL, thermoluminescence.

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on the acceptor side of photosystem II the $Q_B$ binding site is the primary target of photoinhibition.

**Materials and Methods**

*Chlamydomonas stellata*, strain 10-le, Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen, was cultivated autotrophically with CO$_2$ as carbon source as previously described by Wiessner [18]. The alga cultures were cultivated at 10,000 lux during continuous bubbling of the growth medium with a gas mixture containing 5% CO$_2$ and 95% air. Pea (*Pisum sativum* L.) plants were grown in a greenhouse.

The photoinhibitory treatment of alga cells and pea leaves were carried out by exposing them to white light of 300 W/m$^2$ from a 600 W halogen lamp. The alga samples were continuously stirred during illumination and the temperature of the cell suspension was kept at 20 °C. Photoinhibitory light treatment did not cause any change in the chlorophyll content of the cells. Disks of 8 mm diameter were cut from detached pea leaves and on moist filter paper were exposed to light treatment. The samples were protected from the heat effect of light by inserting glass heat filters into the light path. After light treatment the samples were dark adapted for 10 min before oxygen and TL measurements.

Steady state oxygen evolution was measured by using a Clark type electrode in a temperature-controlled cell at 25 °C under saturating white light. The electron transport rate was measured either from water to 500 $\mu$M phenyl-$p$-benzoquinone which can accept electrons from QA [19] or from water to 500 $\mu$M diaminodurene (DAD) and 5 mM ferricyanide. The oxidized DAD accepts electrons at the level of plastoquinone [20]. The chlorophyll concentration of the alga samples was 10 $\mu$g/ml.

Thermoluminescence measurements were carried out in an apparatus similar to that described by Tatake *et al.* [21]. 0.4 ml aliquots of samples containing 125 $\mu$g chlorophyll or small pieces of pea leaves were excited by continuous white light of 10 W/m$^2$ at -80 °C for 30 s. Thermoluminescence was measured at a heating rate of 20 °C/min by an EMI 9558 B photomultiplier. The thermoluminescence glow curves were resolved into component bands by an AT compatible computer using a damped least squares fitting method [22].

**Results and Discussion**

The two main thermoluminescence bands of algae, chloroplasts and leaves are the so-called B and Q bands [17, 23]. The B band originates from radiative charge recombinations occurring between the positively charged S$_2$ state of the water-splitting system and the negatively charged secondary quinone acceptor, $Q_B^-$. The Q band is associated with S$_2$QA$^-$ charge recombination [17, 23]. The areas (or amplitudes) of the bands are proportional to the amounts of S$_2$Q$_B^-$ and S$_2$QA$^-$ redox couples present in the sample after light induced charge separation [17]. Since the S$_2$ state contributes to the generation of both the Q and B bands any difference in the amplitudal change of the bands during photoinhibitory treatment indicate different sensitivity of the QA and Q$_B$ acceptors against photoinhibition.

Fig. 1A shows the resolution of the thermoluminescence glow curve of *Chlamydomonas stellata* into the Q and B bands during photoinhibitory
light treatment. The two additional very small satellite bands applied in our previous work [24] to fit the two sides of the glow curve of *Chlamydomononas reinhardti* at −24 and +45 °C represented less than 1–2% of the total area under the glow curve and therefore they were neglected in the present curve resolution. This simplification is justified by the different excitation conditions. While earlier the thermoluminescence was excited by continuous light during cooling from 20 °C to −60 °C, in the present work excitation occurred at −80 °C only one transition (S1→S2) of the water splitting system is allowed and the two small satellite bands are barely charged. It is also apparent that the fit between the experimentally measured and calculated curves is relatively poor at around zero centigrade due to the distortion caused by the solid-liquid phase transition of water during heating of sample [22]. However, this small fitting error does not influence our conclusions.

The curve resolution in Fig. 1 demonstrates that during photoinhibition in the glow curve of alga cells the amplitude of the B band at 32 °C is diminished faster than that of the Q band at 12 °C. Similar phenomenon was observed by Ohad et al. [12] in an other green alga strain, *Chlamydomonas reinhardti* and by Kirilovsky et al. [15] in *Synechocystis 6714*. Thermoluminescence measurements of pea leaves during photoinhibitory light treatment resulted in an observation similar to that obtained with alga cells. In the glow curve of pea leaf disks the B band at 32 °C was abolished faster than the Q band at 12 °C (Fig. 1 B). The disappearance of the B and Q bands were considerably slower in leaves than in alga cells. This can partly be explained by a decrease of light intensity due to a filtering effect of the upper layers of the leaves.

Decomposition of the glow curves into the Q and B thermoluminescence bands provides exact information about the size of the Qb pool participating in charge separation and recombination. Contrary to this, the amplitude of the Q band obtained in curve resolution does not reflect the total amount of the QA molecules. In the electron transport chains with impaired or permanently reduced Qb acceptors electrons accumulate on QA. However, in the electron transport chains possessing active Qb acceptor the amount of reduced QA molecules is determined by the equilibrium constant, K of the QbQb−⇌QA−Qb equilibrium. Since the value of K is about 16 in the uninhibited electron transport chains only 5–10% of the QA molecules is reduced and participate in the generation of the Q band [25]. However, independently of the electron distribution an increase of the Q band relative to the B band (Fig. 1) indicates a preferential photoinhibitory damage at the Qb site or in agreement with the observation in [26] a permanent reduction of Qb. Simultaneous impairment of the QA and Qb sites would be manifested in a simultaneous abolishment of the Q and B bands [10].

After DCMU addition all of the electrons of the Qb− pool are transferred back to the QA pool and the area (or the amplitude) of the DCMU-induced Q band is proportional to the absolute amount of the QA− molecules. This area is approximately equal to the sum of the Q and B bands in the curve resolution of uninhibited cells (Fig. 1). The amplitudal change of the B band in uninhibited cells and that of the Q band after DCMU addition are shown in Fig. 2 during photoinhibition. Confirming the results of Fig. 1 the impairment of the Qb site (Fig. 2, curve 1, solid line) is faster than that of the QA site (Fig. 2, curve 2, solid line).

The faster decrease of the thermoluminescence B band in comparison to the Q band can be ac-

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**Fig. 2.** Changes in the amplitudes of the B (curve 1) and Q (curve 2) thermoluminescence bands during photoinhibition and dark recovery. *Chlamydomononas stellata* cells were illuminated at 300 W m⁻² (solid lines) and allowed to recover in the dark after 20 min light treatment (dashed lines). The Q band was induced by the addition of 10 μM DCMU after photoinhibitory illumination.
counted for by the combination of three processes. On one hand, during photoinhibitory illumination a part of the Q\textsubscript{B} pool becomes permanently reduced and stores negative charges. These permanently reduced molecules can not participate in charge separation and consequently in charge recombination. This results in a decrease of the B band and due to the accumulation of electrons on Q\textsubscript{A} in a concomitant increase of the Q band. Long-term dark relaxation (oxidation) of the Q\textsubscript{B} pool before thermoluminescence measurement should abolish the phenomenon. A partial reversibility of the B band during dark adaptation of the photo-inhibited alga cells (Fig. 2, curve 1, dashed line) can be explained by the described process. However, the extent of the reversible decrease is negligible in comparison with the irreversible change in the amplitude of the B band suggesting the contribution of damaging mechanisms, as well. A partial recovery of the B band was also observed in Chlamydomonas reinhardtii [12] and in spinach thylakoids during dark adaptation after photoinhibitory light treatment [27]. Consistently with the TL observations a reversible disappearance of variable fluorescence [14] and oxygen evolution [28] was also observed in Synechocystis and in spinach thylakoids, respectively. In alteration from the B band the Q band is diminished almost irreversible during photoinhibition (Fig. 2, curve 2, dashed line). The lack of a partial recovery in the amplitude of the Q band can be accounted for by a fast oxidation of the reduced Q\textsubscript{A} acceptor molecules either by transferring electrons to Q\textsubscript{B} or undergoing backreaction with the donor side following photoinhibitory light treatment. We note that in Fig. 2 the reversibility of photoinhibition is shown only after 20 min of light treatment because after 40 min illumination both the Q and B bands are irreversibly diminished.

The second possible explanation of the loss of the B band is that during photoinhibition the number of active Q\textsubscript{B} binding sites decreases due to an alteration or degradation of the carrier protein [12]. A gradual irreversible loss of the Q and B bands during photoinhibitory light treatment substantiates that a degradation process also contributes to the abolishment of thermoluminescence emission. The damage of Q\textsubscript{B} site due to overexcitation of PS II can be induced by the formation of reactive semiquinone anion radical (Q\textsubscript{B}\textsuperscript{-}) either directly or mediated via an oxygen or hydroxyl radical [1, 13].

A third process which may also contribute to the faster disappearance of the B thermoluminescence band can be based on the heterogeneity of PS II reaction centers [29, 30]. The PS II\textsubscript{β} centers which are incapable of reducing the plastoquinone molecules (Q\textsubscript{B}-nonreducing center) are very resistant to photoinhibition [7, 31]. This phenomenon can be similar to the slight protection provided by DCMU against photoinhibition [2, 13, 32]. It can be assumed that during photoinhibitory light treatment the preferential damage of Q\textsubscript{B}-reducing PS II\textsubscript{α} centers in comparison to the Q\textsubscript{B}-nonreducing PS II\textsubscript{β} centers can contribute to a faster decrease of the B thermoluminescence band relatively to the Q band. The Q\textsubscript{B}-nonreducing PS II\textsubscript{β} centers are located in the non-appressed stroma thylakoid regions. The appressed granal regions contain Q\textsubscript{B}-reducing PS II\textsubscript{α} centers [29, 30]. Since in vitro systems contain more stroma region than the in vivo isolated thylakoids the phenomenon may be more apparent in in vivo than in in vitro samples.

As shown in Fig. 1 A (by vertical lines) in alga cells the peak positions of the Q and B TL bands are not shifted during photoinhibitory light treatment. This suggests that the redox potential of the primary and secondary quinone acceptors are not changed during photoinhibition. Similar conclusion was reached on the basis of TL investigation of Synechocystis [15]. However, in Chlamydomonas reinhardtii the peak position of the B band was shifted towards lower temperature during photoinhibition indicating a modification in the midpoint redox potential of Q\textsubscript{B} [12, 13]. Similarly to the alga cells in the glow curve of pea leaves the peak positions of the Q and B bands were not influenced by photoinhibition (Fig. 1 B).

Partial electron transport rate measurements were in good correlation with the TL measurements. In Chlamydomonas stellata the Hill reaction rate from water to oxidized diaminodurene (an acceptor at the level of plastoquinone) decreased faster than from water to phenyl-p-benzoquinone (electron acceptor after Q\textsubscript{A}) (Fig. 3). These results are in agreement with earlier observations [1, 2]. During photoinhibitory light treatment the Q\textsubscript{B}-dependent electron transport rate from water to 2,6-dichlorophenol-indophenol decreased more rapid-
Fig. 3. Effect of photoinhibitory light treatment on the electron transport rate of autotrophic *Chlamydobotrys stellata* cells measured either from water to 500 µM phenyl-p-benzoquinone (○―○) or from water to 500 µM diaminodurene plus 5 mM ferricyanide (●―●). The absolute rates of oxygen evolution in untreated samples were 150 ± 9 µmol O₂/mg Chl h (○―○) and 120 ± 7 µmol O₂/mg Chl h (●―●). Data points are the average of three measurements. Standard deviations of data are represented by bars.

ly than the Q₈-independent (from water so silicomolybdate) electron transfer. Similarly to the interpretation of the thermoluminescence measurements the faster decrease of the Q₈-dependent electron transport rate relatively to the Q₈-independent rate can be accounted for by the development of photoinhibition at the Q₈ binding site [1, 2, 32] and by a differential degradation of the Q₈-reducing and Q₈-nonreducing centers [7, 31].

In conclusion we can say that in *Chlamydobotrys stellata* cells and in pea leaves at high light intensities photoinhibition is initiated at the Q₈ site. Partial electron transport rate measurements [1, 2, 33] and TL observations [13–15] obtained with other alga strains support this conclusion. Moreover, an almost complete loss of Q₈-dependent PS II activity was accompanied only with a 20% loss of signal II (tyrosine Z⁺) suggesting a faster damage at the acceptor side than at the donor side of PS II [2]. Our conclusion is also consistent with the recent hypothesis that the occupancy of the Q₈ site may control the photoinactivation of PS II [13, 32, 34]. However, in alteration from the *in vivo* systems in isolated spinach thylakoids [10] and pea chloroplasts [35] the Q and B thermoluminescence bands were simultaneously abolished indicating that the primary (Q₈) and secondary (Q₈) acceptors are simultaneously damaged.

Recent reports indicate that the damage during photoinhibition is not restricted to a single site but depends on the excitation light intensity. At high light intensities a propagation of damage may occur. Following the reduction of Q₈ pool the stable charge separation between P680⁻ and Q₈⁻ can also be impaired [7, 9, 36]. A possible mechanism for this photoinactivation is a double reduction and subsequent protonation of Q₈ leading to a loss of the Q₈H₂ from the binding site [6, 37]. In the absence of Q₈⁻ the formation of P680 triplet is facilitated [37] which in turn can mediate the formation of reactive singlet oxygen and membrane damage [38].

A different mechanism of photoinhibition is proposed to be dominant at low light intensities. It has been suggested that at low light conditions the D1 turnover and inhibition of electron transport are controlled by formation of oxidizing species on the donor side of PS II [3–5, 39]. Adapting the idea of Krieger and Weiss [40], according to which the formation of high ΔpH induces Ca²⁺ release from the watersplitting system a mechanism of donor side photoinhibition can be proposed. After the ΔpH induced release of Ca²⁺ the retarded electron donation from the watersplitting system may cause the accumulation of long-lived His⁺ [41, 42], Tyr⁺ [3–5] and P680⁺ radicals which in turn may destroy the *in vivo* structure of the D1 protein. Although, we have to mention that in contrast to this expectation high ΔpH provided some protection against photoinhibition [43].

Taking together the present observations with the literature of photoinhibition we can say that the dominant mechanism and the site of most apparent photoinhibitory damage greatly depend on the illumination conditions. The conclusion of the present work is that in *in vivo* system at high light intensities first the normal action of the Q₈ binding site is inhibited during photoinhibition.

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