In Pea Thylakoids, a Special Thermoluminescence Band Was Observed at Low Temperature after Photoinhibitory Treatments Performed under Aerobic Conditions, either *in vivo* (in Leaf) or *in vitro*

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Characteristics of thermoluminescence (TL) glow curves were studied in thylakoids isolated from pea leaves after an exposure to very high light in the TL device. The inhibition of photosynthesis was detected both as decreases of oxygen evolution rates and of variable fluorescence. In another more classical experiment, leaves were exposed to high light at low temperature (5 °C) for 4 h and recovery of normal PS II characteristics were followed during a 20 h time period under low light at 20 °C. TL study was performed on thylakoids isolated from these leaves. Charging of bands was performed by an illumination at low temperature (−80 °C).

Illumination at low temperature (−80 °C) induces 2 types of TL bands called Z variable (Z_v) band peaking at about −70 °C and a classical B band peaking at 35 °C. B band is ascribed to recombination in pairs $S_{2/3}Q_B^-$, whereas the origin of Z_v bands remains unclear. Modified Z_v bands, with flat shape and presenting a large part resistant to ethanol, were evidenced after some minutes of dark adaptation, both in light-exposed thylakoids and in thylakoids isolated from photoinhibited leaves. This ethanol resistant part in Z_v band was only induced by photoinhibitory treatment performed under aerobic conditions (21% O_2 in media), both *in vitro* and *in vivo;* thus, the appearance of the special Z_v band would be related to an oxidative process accompanying photoinhibition. B bands presented reduced sizes and a shift of peak maximum of about 5 °C after some minutes of dark adaptation. *In vivo,* the recovery of a high PS II activity and of B bands displaying normal characteristics occurs parallely to reappearance of Z_v bands with normal shape and with a vestigial ethanol resistant band.

Introduction

Exposure of thylakoids to high light intensity, far in excess to that required to saturate photosynthesis, results in a decrease of PS II activity known as photoinhibition of photosynthesis (review in [1]). Photoinhibition can be induced *in vivo* when an illumination is applied to leaves at chilling temperature, *i.e.* in conditions where photosynthetic activity is slowered whereas absorption of light remains maximum (review in [2]). It was shown to occur in field conditions, in nordic countries, where association of high light and low temperature is encountered in the morning time; recovery of normal PS II activity can be achieved in some hours: 8–10 h [3]. Photosystem II is the primary target of the photoinhibitory process; mechanisms at the origin of the PS II impairment would include damages at the acceptor side, probably at the level of the Q_B niche on the D_1 protein [4] which could lead to double reduction of Q_A on the D_2 protein [5]. Impairment of the primary charge separation, as well as damages at the donor side, would occur in a further step, but seem excluded in the experimental conditions described above. Only a few studies have been performed with higher plants grown in field conditions: see for instance experiments on spinach in [6]. In laboratories conditions, photoinhibition is currently realized by exposing suspensions of algaes or of isolated thylakoids to high light at room temperature (or near 0 °C), in the absence of electron acceptor. Photoinhibition can be detected as a decrease in oxygen evolution rates in the presence of an electron acceptor, at light saturation. The process is also characterized by a decrease in the variable fluores-

Abbreviations: chl, chlorophyll; F_o, initial fluorescence; F_v, variable fluorescence; F_p, fluorescence maximum; pBQ, *p*-benzoquinone; PPFD, photosynthetic photon flux density; PQ, plastoquinone; PS II, photosystem II; Q_A, primary electron acceptor of PS II; Q_B, secondary electron acceptor of PS II; TL, thermoluminescence; Z_v band, Z (variable) TL band.

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cence, F_v, of PS II, expressed as the ratio F_v/F_m (F_m: maximal fluorescence of PS II), stable for minutes (to hours) in the dark [3, 7].

Thermoluminescence (TL) is a useful technique for monitoring the redox state of functional groups at the acceptor and donor sides of PS II (reviews in [8–10]). Charging of TL bands can be performed either by actinic flashes fired at ambient temperature or by an illumination at low temperature (near −80 °C). In this latter condition, it is possible to detect very unstable charge pair species with rapid recombination at ambient temperature [10, 11].

In the following paper, we present a comparative study of the effects of high light applied to isolated thylakoids and to intact leaves, on the TL properties of the two types of material. We have especially followed kinetics of changes in characteristics of TL bands induced by an illumination given at low temperature in function of the length of the dark adaptation period following the photoinhibitory treatment.

Materials and Methods

Plant material

Pisum sativum var. Merveille de Kelvedon was grown on vermiculite supplemented with nutrition medium in a partially shaded green house: illumination was provided by a combination of solar and artificial light (Osram lamps HQIL 400 W) giving an average photosynthetic photon flux density (PPFD) of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ during a 14 h photoperiod. Temperature was controlled at 22 ± 0.5 °C day/17 ± 0.5 °C night.

Isolation of chloroplasts and measurement of electron transport activity

Intact chloroplasts were isolated from fully expanded leaves of 3-week-old plants using the method of Cerovic and Plesnicar [12]; the degree of intactness was about 85%. The chloroplasts were osmotically broken by suspension in 5 mM $MgCl_2$ for 30 s then resuspended in the following medium: 350 mM sorbitol, 5 mM $MgCl_2$, 5 mM KCl, 25 mM Hepes (pH 7.5) at chlorophyll concentration of 40 $\mu g \cdot ml^{-1}$. Aliquots of 0.6 ml were centrifuged in Eppendorff minivials and the pellets kept in the dark, on ice before the TL analysis. Electron transport was measured polarographically as O_2 evolution rates using a Clark type electrode (Hansatech) at controlled temperature of 22 °C; the suspension buffer contained in addition 1 mM pBQ and 1 μM nigericin; rates of uncoupled rates of O_2 evolution were in the range 350–500 μmol/μg chl per h.

Photoinhibitory treatments

1) Treatments in the TL apparatus

Thylakoids were subjected directly in the cuvette of the TL device, to illuminations leading to photoinhibition. A pellet was resuspended in 350 μl of the buffered medium described above and the suspension poured in the cuvette. Samples were illuminated at light intensity of 9000 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 90 s at 20 °C in an O_2 deprived medium, obtained by bubbling pure argon for 30 s in the suspension before the illumination period. For one experiment, flushing with argon was for 20 s and followed by another flushing with pure O_2 in the suspension. At the end of the illumination period, samples were centrifuged in Eppendorff minivials (30 s, 2000 × *g*) and the pellets kept on ice for 5 min. For the study of Z_v bands, since glycerol destroys this type of TL band, at least in the control, resuspension of thylakoids was in the buffered medium.

2) Treatments in transparent chamber

One leaf (2 leaflets) of a pea plant was enclosed in a thermocontrolled transparent chamber flushed with humidified air (RH 80%) and exposed to white light at PPFD 1200 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 4 h at 5 °C. For some experiments air was replaced by pure nitrogen or a mixture of N_2–O_2 (98:2, vol/vol). At the end of the photoinhibitory treatment, the plant was first left in the dark at 20 °C for about 40 min in order to determine changes in fluorescence parameters (see later), then the leaf was either kept in the dark or illuminated with light of low intensity (20 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 20 h. The TL study was performed on thylakoids isolated from one leaflet, as explained above, the other leaflet being kept as a control.

Fluorescence measurements

Induction curves of fluorescence were recorded directly on leaves of a plant using a home-made

device composed of a Hansatech detector + amplifier linked to an Apple II computer [13]; the computer allows calculation of the parameters F_o and F_p of the fluorescence induction curve (F_o, initial fluorescence; F_p, fluorescence maximum); F_v, variable fluorescence was calculated as $F_v = F_p - F_o$. Actinic light was delivered by a HeNe laser giving monochromatic light (λ = 632 nm); PPFD was 120 $\mu M \cdot m^{-2} \cdot s^{-1}$ at the level of the sample and area exposed to light was about 1 cm^2. The leaves were usually dark-adapted for about 10 min, then exposed 4 s to laser light for fluorescence measurements. Using a modulated fluorimeter (Walz), we checked that in our experimental conditions, F_p was about 95% of F_m, the true maximal fluorescence; thus we considered $F_p \sim F_m$. Fluorescence measurements with isolated thylakoids were performed directly in the TL cuvette using the same device.

Thermoluminescence assays

The TL device has been described in previous publications [6, 14]. TL curves were recorded in the range −110 to +70 °C, with heating rate of 0.5 °C/s. Charging of TL bands was performed by illumination of the sample at −80 °C using red light ($\lambda > 650$ nm, 3000 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 5 s). Each curve presented in figures corresponded to Fourrier-filtering of the data points [6] for one experiment.

Results and Discussion

Photoinhibition in vivo and in vitro; determination of PS II activities

Using a classical protocol, photoinhibition was induced in pea leaves by a 4 h exposure to high light (PPFD of 1200 $\mu E \cdot m^{-2} \cdot h^{-1}$) in a chamber thermocontrolled at 5 °C flushed with air. Recovery from photoinhibition was followed during a period of about 20 h at 20 °C under low light intensity. The ratio F_v/F_p (report to: *Fluorescence measurements* in Materials and Methods), determined on the upper part of the intact leaf, was equal to 0.8 in dark-adapted (DA) control leaf; F_o was determined simultaneously. The ratio appeared decreased to 0.25–0.35 when measured after a 10 min dark adaptation period following the photoinhibitory treatment; when the leaves were maintained in the dark for a longer period, an increase of the ratio was first observed to a value of 0.4–0.45 which stabilized after 30 min and kept constant as long as the leaf remained in the dark (Fig. 1). Such a decrease in the ratio F_v/F_p is considered as a proof for occurrence of photoinhibition of photosynthesis [3, 7]. When the photoinhibited leaf was submitted to an illumination with light of low intensity and at 20 °C, recovery to the initial value of 0.8 occurred in 10–12 h with a half-time of 3 h (Fig. 1). F_o was about 15% increased after the photoinhibitory treatment and a normal value was recovered in a 1 h period of illumination under low light intensity (not shown).

Very similar results were obtained when air was replaced by a mixture of N_2–O_2 (80:20) and the leaves exposed to strong light for only 2–3 h (not shown). When pure N_2 was flushed on illuminated leaves, F_v/F_p decreased quickly (in 30–40 min) to low values; recovery of initial values of the ratio was slow ($t_{1/2}$ 4–5 h) and in most cases incomplete (not shown).

Isolated thylakoids were submitted to high light directly in the TL cuvette in the absence of electron acceptor. In O_2 deprived medium, exposure for 90 s to PPFD of 9000 $\mu E \cdot m^{-2} \cdot h^{-1}$ at 20 °C produced a 35–40% inhibition of PS II activity (de-

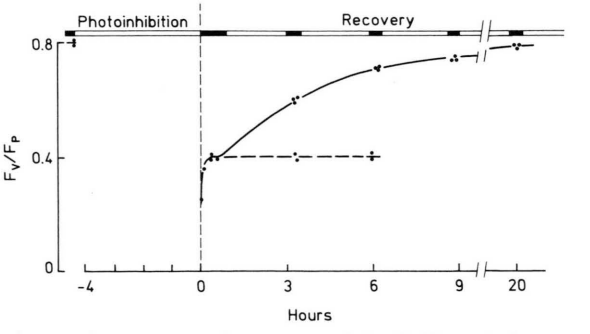

Fig. 1. Time course of recovery of the F_v/F_p ratio in pea leaf after a photoinhibitory treatment. Two leaflets of a leaf (maintained in relation to the plant) were enclosed in a thermocontrolled transparent chamber flushed with air and exposed to high light (PPFD 1200 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 4 h at 5 °C. At the end of this treatment, the leaf was maintained in the dark at 20 °C for 40 min to determine the initial value of the F_v/F_p ratio and its variation in the dark; then, the leaf was either kept in the dark for 6 h (broken lines) or exposed to low light intensity (20 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 20 h at 20 °C. In the course of this latter period, after 3, 6, 9 and 20 h, F_v/F_p ratios were determined: 3 determinations, 2 min apart after 10 min darkness. Similar measurements were performed before the exposure to high light.

termined as O_2 evolution rates in the presence of *p*-benzoquinone as electron acceptor). In the same thylakoids F_v/F_p was decreased to 0.50 with a 15% increase of the F_o level; this ratio remained stable in the dark for at least a 1 h time period. These results indicate the occurrence of a photoinhibitory process.

TL glow curves in thylakoids charged by an illumination at −80 °C

Thylakoids were either directly exposed to high light in the TL device, or isolated from leaves which were submitted to the classical photoinhibitory treatment previously described in Fig. 1; thylakoids *in situ* were able to recover a normal PS II activity after a 20 h illumination period at low light intensity.

1) Thylakoids submitted to high light

An illumination at −80 °C (to charge bands) induces 2 types of TL bands: B type bands, ascribed to recombinations in $S_{2/3}Q_B^-$ pairs (pairs $S_2Q_A^-$ are formed at this low temperature and electrons are transferred to Q_B/Q_B^- at temperatures above −60 °C [15]) and Z_v (Z variable) type bands. The latter bands, of small size, are peaking near −68 °C in dark-adapted control thylakoids, and oscillate with a periodicity of four, when thylakoids are preilluminated by flashes fired at ambient temperature [11]. They would correspond to recombinations in pairs "S_2" Q_A^-, "S_2" being an unstable S_2 state [15]. Ethanol (or glycerol) suppresses about 90% of the Z_v band, leaving a vestigial band presenting the same shape: Fig. 2, curve 3. We previously showed that a photoinhibitory treatment performed in the absence of electron acceptor on isolated thylakoids suspended in a normally aerated medium, induced the appearance of a new Z_v band resistant to ethanol and with a relative size related to the degree of photoinhibition [17]. Such a band did not oscillate when the thylakoids were preilluminated by flashes at room temperature [17]. When photoinhibition was carried out under anaerobic conditions (in medium pregassed with argon), and illumination by red light to charge bands performed after 5 min dark adaptation, the size of the Z_v band was reduced, as was that of the B band. Characteristics of the B bands were studied in another publication (J. Farineau, submitted). The Z_v band appeared to be sensitive to ethanol; a residual resistant emission still re-

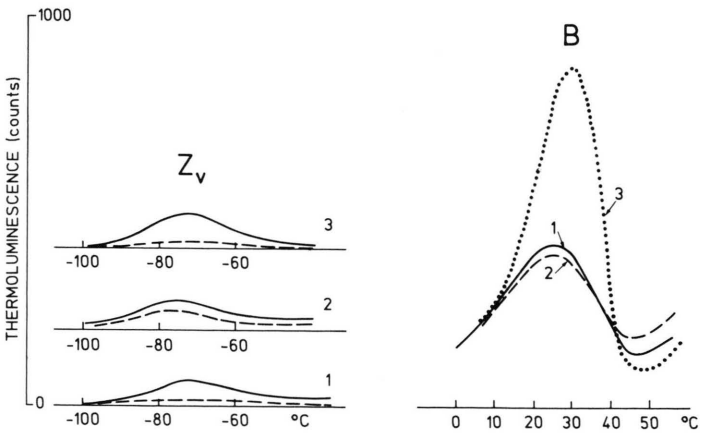

Fig. 2. TL glow curves of thylakoids induced by an illumination at −80 °C (5 s continuous light, $\lambda > 650$ nm, 3000 $\mu E \cdot m^{-2} \cdot s^{-1}$). Units in ordinate are counts numbers determined by the photon counting device. For reasons of clarity, the Z_v bands (in range −100 to −50 °C) and the B bands (in range +10 to +50 °C) are presented separately. In Z_v bands, the vestigial component observed after addition of 2.5% ethanol is indicated by broken lines. Isolated thylakoids were submitted to 2 min high light (9000 $\mu E \cdot m^{-2} \cdot s^{-1}$), in the TL device. In 1) the medium was got rid of oxygen by flushing argon for 30 s; 2) is a similar experiment except that pure O_2 was bubbled for 10 s after 20 s of argon flushing; 3) is a control with argon flushing but no exposure to high light. After illumination, the thylakoids were centrifuged and the pellet kept 5 min in darkness, on ice; then, the thylakoids were rapidly resuspended in the buffer medium. The B bands which are presented are those observed after addition of 2.5% ethanol, which suppresses a large emission at 50–60 °C seen only in photoinhibited material.

mained (Fig. 2, curve 1) which corresponded to a baseline or possibly to the tail of a Z band [9]. When after a short gassing by argon, pure O_2 was bubbled in the suspension, the photoinhibitory treatment performed in the same conditions induced a larger Z_v band while B bands kept nearly similar sizes in the 2 experiments (in the presence or the absence of O_2). In the presence of ethanol the residual band observed in aerobic conditions was of much larger amplitude than in anaerobiosis: Fig. 2, curve 2. These results demonstrate that oxygen induces a modification of the properties of either "S_2" or more probably of Q_A^- (or of special molecules in the environment of Q_A^-); nevertheless these changes accompanying photoinhibition would be additional effects which differ from the processes leading to impairment of photosystem II.

2) Thermoluminescence glow curves in thylakoids isolated from photoinhibited leaves

Leaves were illuminated for 4 h (PPFD 1200 $\mu E \cdot m^{-2} \cdot s^{-1}$) in an atmosphere of air, at 5 °C. Thylakoids were rapidly isolated (in 5 min) from leaves just after the end of the photoinhibitory treatment, or from identical leaves postilluminated at low light intensity for 3 or 20 h, for recovery of partial or full PS II activity. Control thylakoids were isolated from dark-adapted untreated plants. Charging of bands was performed by 5 s illumination with red light at −80 °C.

Study of B bands. TL B bands were observed in thylakoids isolated from leaves 10 min after the end of the photoinhibitory treatment; they were of low amplitude with a shift of the peak maximum of about −5 °C (Fig. 3, curve 1) as compared to the large B band observed in the control peaking at 36 °C (not shown). Low amplitude of the band in photoinhibited material is related to the degree of inhibition of the PS II activity observed (confirming results in [17, 18]): 68% of that of the control (activity was expressed as rates of O_2 evolution in the presence of *p*-benzoquinone). When these thylakoids were kept 1 h in the dark on ice, the bands appeared shifted by +2−3 °C (not shown). When leaves were postilluminated for 3 h at low light intensity, the recovery of 52% of PS II activity in thylakoids was accompanied by a large increase of the B band; its maximum was at the same temperature as in the control, 36 °C (curve 2). Finally, recovery of band B with normal size was achieved after 20 h leaf postillumination (curve 3).

Study of the Z_v bands. In the control thylakoids, the Z_v bands were typical with a nearly sharp shape, a peak maximum near −72 °C and no emission at −90 °C [17]. The residual band observed after ethanol addition exhibited the same characteristics (not shown). In thylakoids isolated in less than 10 min after the photoinhibitory treatment, the Z_v band was large, of flat shape with peak maximum near to −70 °C and some emission remaining at low temperature: −100 °C. Addition of ethanol left a large flat band: its area was about 50% that of the band in untreated material and the maximum was shifted to about −80 °C (Fig. 3, curve 1). Thylakoids isolated from photoinhibited leaves postilluminated for 3 h presented Z_v bands having recovered a normal shape; the residual ethanol-resistant band had a relative area intermediary between those observed in photoinhibited

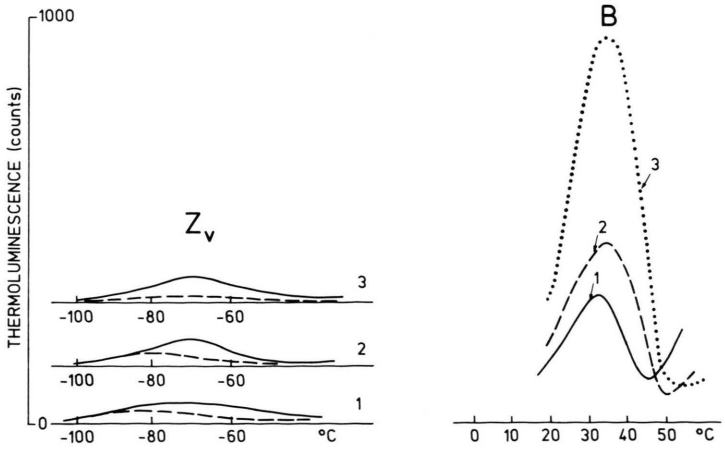

Fig. 3. Same experiment as in Fig. 2 with thylakoids isolated from pea leaves which have been submitted to the photoinhibitory treatment described in the Fig. 1. Curve 1: thylakoids isolated in the dark (in 5 min), after the photoinhibitory treatment and TL curves determined 2 min after (inhibition of PS II activity was 68%); curve 2: plants were exposed 3 h to low light in order to obtain some recovery of PS II activity (inhibition of 48%); curve 3: 20 h of exposure to low light (total recovery of PS II activity).

material and in control thylakoids; it was still of flat shape with maximum near −80 °C (curve 2). After a 20 h postillumination leading to total recovery of PS II activity, the sizes and shapes of the Z_v bands (normal and residual band in the presence of ethanol) were identical to those observed in control thylakoids (curve 3).

In addition we confirmed results obtained *in vitro:* no ethanol-resistant band was observed in leaves photoinhibited in an atmosphere of pure N_2 (not shown).

In conclusion, in photoinhibited leaves, recovery of normal Z_v bands with disappearance of the ethanol-resistant contribution, accompanied the recovery of B bands with normal characteristics, *i.e.* peak maximum at high temperature (at about 35 °C) and large amplitude.

Conclusion

When isolated thylakoids were submitted to photoinhibition in an oxygenated medium, a special Z_v band resistant to ethanol was evidenced; we showed that it was not observed in the complete absence of oxygen. In this respect, thylakoids extracted from leaves photoinhibited in an oxygenated medium (and studied after only some minutes of dark adaptation) and isolated thylakoids directly exposed to high light had exactly the same properties. The ethanol-resistant Z_v band disappeared during the recovery of a normal PS II activity in leaves. Thus, it should originate from inactive PS II centers, unable of any oxygen evolution. This special Z_v band could result from the action of oxygen radicals on PS II, which could be formed in thylakoids *in vivo* as well as *in vitro,* in the conditions of our experiments, *i.e.* under high light, in the presence of oxygen and in the absence of electron flow [19]. The target of these radicals could be lipids which are known to be present in the environment of the Q_A site [20]. The suppression by ethanol of Z_v band is sometimes interpreted in a similar way, as due to an effect of the alcohol on lipids present in the Q_A vicinity [10].

Acknowledgements

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