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Effect of complete extraction and re-addition of manganese on thermoluminescence of pea Photosystem II preparations

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Thermoluminescence of Photosystem II particles isolated from pea chloroplasts using digitonin and Triton X-100 was measured after 1 min illumination at a certain temperature (T_{ex}) followed by illumination during cooling (40 Cdeg/min) to a lower temperature. Glow curves of the particles are characteristic of the photosynthetic oxygen-evolving material studied earlier. Complete (more than 95%) removal of Mn from the Photosystem II particles abolishes thermoluminescence bands around 0°C, related to the oxygen-evolving system, but the thermoluminescence bands peaking around −30°C (TL_{-30}), −55°C (TL_{-55}) and between −68 and −85°C, depending on T_{ex}(TL_v), remain unaltered. The bands are characterized by different dependence on T_{ex}. The TL_{-30}, TL_{-55} and TL_v bands can also be observed in the glow curve of isolated pea and spinach chloroplasts. Re-addition of $MnCl_2$ (2 μM, corresponding to nearly 4 Mn atoms per reaction center of Photosystem II) to the Mn-depleted particles does not reactivate the thermoluminescence bands around 0°C. However, it does lead to suppression of TL_{-30} accompanied by parallel activation of TL_{-55}, revealing competition of the TL_{-30} and TL_{-55} for charges generated by the reaction center. These data, as well as the results on the effect of inhibitors and electron donors to Photosystem II, show that positive charges contributing to the TL_{-30}, TL_{-55} and TL_v thermoluminescence bands are located on secondary electron donors of Photosystem II which do not require Mn and are located closer to the reaction center than the Mn-containing, water-oxidizing enzyme.

Introduction

Thermoluminescence (TL) of green plants is characterized by at least six glow peaks emitting at different temperatures and denoted as Z (−160°C), Z_v(variable depending on excitation temperature), A(−10°C), B_1(+25°C), B_2(+40°C) and C(+55°C) bands [1–13]. Bands A, B_1 and B_2 are closely related to the photochemical reactions of Photosystem II (PS II) responsible for oxygen evolution [6–8]. The intensity of the three bands undergoes a period-four oscillation when excited in a sequence of flashes [10,12,14,15]. The treatment of chloroplasts with Tris, leading to partial (60–70%) extraction of Mn, as well as heat treatment abolish the B_1 and B_2 bands, leaving the A band unchanged [6,8,16]. However, the effect of complete removal of Mn on thermoluminescence has not been previously studied. Recently, a procedure of 'complete' (more than 95%) extraction of

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol, DPC, diphenylcarbazide; PS II, Photosystem II; TL, thermoluminescence; Chl, chlorophyll; RC, reaction center.

Mn from PS II preparations has been reported [17]. Restoration of PS II activities after the treatment can be achieved by addition of catalytic quantities of $MnCl_2$ (approx. 10^{-7} M). Here we report data on the effect of complete extraction and re-addition of Mn on thermoluminescence of PS II preparations.

Materials and Methods

DT-20 subchloroplast enriched in PS II were prepared by treatment of pea chloroplasts with digitonin (0.4%) and Triton X-100 (0.1%) followed by centrifugation (30 min at $20000 \times g$) as in Ref. 17. Content of PS II in the particles was 1 RC/200–220 Chl molecules; the Hill reaction rate evaluated from both photoreduction of DCIP and O_2 evolution in DT-20 subchloroplast particles was approx. 20% of that observed in chloroplasts [17]. The 'complete' (more than 95%) extraction of Mn from DT-20 subchloroplast particles was reached as described in Ref. 17. The DT-20 subchloroplast particles at 50 μg Chl/ml were incubated for 1 h at 2°C in a medium containing 1 M Tris-HCl (pH 8.0) as well as 0.5 M $MgCl_2$ followed by precipitation at $20000 \times g$. The pellet was washed twice by resuspending in 10 μg Chl/ml, and centrifuged, first in 0.8 M Tris-HCl (pH 8.0) then in a medium comprising 20 mM Tris-HCl (pH 8.0)/35 mM NaCl. The untreated and 'completely extracted' DT-20 subchloroplast particles were frozen in the latter medium, which additionally contained 10% glycerol, and were kept in liquid N_2 at Chl concentration of about 2 mg/ml. Before the experiments, samples were thawed and diluted in the medium containing 50 mM Hepes-NaOH buffer (pH 7.5) and 35 mM NaCl to a Chl concentration of 100 μg/ml; 0.5 ml aliquots of the suspension were used for thermoluminescence measurements. Spinach and pea chloroplasts were isolated as described in Ref. 18.

Thermoluminescence was measured in the temperature region from −100°C to +90°C by using the apparatus described earlier [9,12] similar to that of Sane et al. [5]. The light emission was measured by a red-sensitive photomultiplier (EMI 9558 B) and the signal was amplified through a home-made differential amplifier and fed to an *X-Y* recorder. The temperature of the sample holder was monitored using a platinum resistor thermometer placed below the samples. The samples were illuminated with white light from a Narva halogen lamp of 650 W. The exciting light was passed through a heat-absorbing water filter (thickness 10 cm) and a Balzers neutral density filter giving an illumination intensity of 5 W/m^2. The samples were excited for 1 min at indicated temperatures, then were cooled to −100°C during illumination at a cooling rate of 40 Cdeg/min. After excitation of the samples, thermoluminescence measurements were performed at a heating rate of 20 Cdeg/min. The dips on glow curves (see Fig. 1) around 0°C are artifacts due to the melting of ice (all the experiments were performed without addition of glycerol).

Results and Discussion

Thermoluminescence of untreated PS II particles

The glow curves of untreated subchloroplast PS II particles DT-20, excited by 1 min illumination at +5°C followed by illumination during cooling to −100°C, are characteristic of thermoluminescence of oxygen-evolving preparations [1–3,5–12]. These possess dominating bands between −20 and +50°C, corresponding to the A and B bands, and the C band around +65°C (Fig. 1). A band peaking at around −30°C (TL_{-30}) appears when excitation starts from −30°C, and a new thermoluminescence band with an emitting peak around

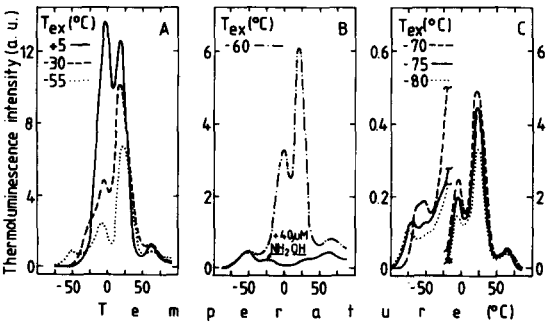

Fig. 1. Glow curves of untreated subchloroplast PS II particles DT-20, excited by 1 min illumination at indicated temperature (T_{ex}) followed by illumination during cooling (40 Cdeg/min) to −100°C. The subchloroplast PS II particles were suspended in a medium comprising 50 mM Hepes (pH 7.5)/35 mM NaCl. In part C, the left scale is for all curves in the temperature range from −100 to −20°C and the right scale is related to the temperature range from −20°C to +100°C.

$-55°C$ (TL_{-55}, seen simultaneously with the TL_{-30} band) is revealed after excitation at temperatures between -50 and $-60°C$ (Fig. 1A). Further lowering of the excitation temperature (T_{ex}) leads to the appearance of a new thermoluminescence band (TL_v) on the left slope of the TL_{-55} band. When the PS II particles are excited at $-75°C$, the three thermoluminescence bands (TL_{-30}; TL_{-55} and TL_v) can be simultaneously observed in the glow curve (see solid curve in Fig. 1C). Upon lowering of the T_{ex} from -60 to $-80°C$, the intensity of the TL_{-55} band is considerably decreased, though the peak position located around $-55°C$ is unchanged. The peak position of the TL_v is shifted from around -68 to $-85°C$ and its intensity is decreased by a factor of 2–3 when the T_{ex} is lowered from -70 to $-90°C$.

Addition of an inhibitor of the oxygen-evolving system, NH_2OH (40 μM) abolishes the intensive glow peaks around 0°C, but the low temperature bands (TL_{-30} and TL_{-55}) as well as the C band (at $+65°C$) remain (Fig. 1B).

Effect of complete removal of Mn

The 'complete extraction' of Mn leads to an effect similar to that caused by NH_2OH treatment. The glow peaks around 0°C are completely eliminated, while the bands TL_{-30} and TL_{-55}, as well as another intensive band at around $+65°C$, remain. In addition, two smaller bands around $+15$ and $+35°C$ can also be observed in the glow curve (Fig. 2). The 'uncovering' of the hidden TL_{-30} and TL_{-55} bands due to inhibition of the glow peaks around 0°C related to oxygen-evolution (Fig. 2) allows a more explicit, comparative investigation of the low-temperature thermoluminescence bands.

The two bands (TL_{-30} and TL_{-55}) are characterized by quite different dependence on T_{ex} (Figs. 2 and 3). The efficiency of charging of the TL_{-30} band is high when it is excited between -30 and $+5°C$ and it decreases rather sharply at T_{ex} below $-30°C$ (resulting in a 50% signal at $T_{ex} = -55°C$ and one about 10% at $T_{ex} = -80°C$). The emission peak of TL_{-30} is located around $-26°C$ when excitation starts at $+5°C$, and around $-30°C$ at $T_{ex} = -30°C$, it remains between -30 and $-35°C$ when T_{ex} is lowered to $-80°C$.

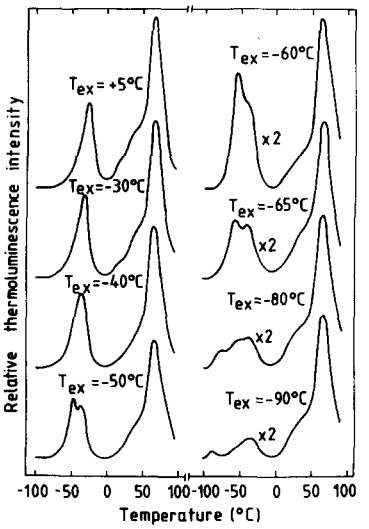

Fig. 2. Glow curves of the 'completely extracted' subchloroplast PS II particles DT-20 (after complete removal of Mn), excited by 1 min illumination at indicated temperature (T_{ex}) followed by illumination during cooling (40 Cdeg/min) to $-100°C$.

TL_{-55} is efficiently charged in a very narrow temperature region (Fig. 3). The efficiency of charging of the TL_{-55} band sharply decreases below $-60°C$ (parallel with the decrease of TL_{-30}) and above $-50°C$ (parallel with the increase of TL_{-30}). Apparently, there is a replacement of the TL_{-30} band by the TL_{-55} band when T_{ex} is lowered from -30 to $-60°C$. A similar effect,

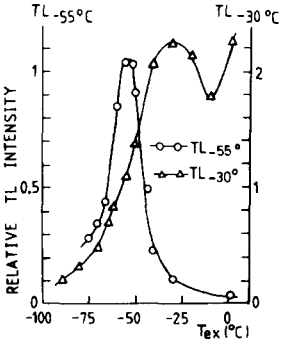

Fig. 3. Dependence of amplitude of the thermoluminescence bands appearing at around $-55°C$ (TL_{-55}) and at around $-30°C$ (TL_{-30}) in the 'completely extracted' subchloroplast PSII particles DT-20 on excitation temperature (T_{ex}). (For details on excitation conditions see Fig. 2.) Intensity of TL_{-55} was measured in the presence of 2 μM $MnCl_2$ when intensity of the band at $-30°C$ was essentially abolished (see Fig. 4C).

namely: a decrease in the charging of the TL_{-55} band accompanied by the appearance of the TL_v band is observed upon lowering T_{ex} below $-60°C$ (Figs. 1 and 2).

The following observation in the interrelation between TL_{-55} and TL_{-30} is of interest. TL_{-55} is not charged when excitation starts at $-30°C$ although the sample is also illuminated at temperatures between -50 and $-60°C$ (favourable for the charging of the TL_{-55} band) during subsequent cooling. Consequently, pre-charging of TL_{-30} evidently prevents charging of TL_{-55}.

Effect of addition of $MnCl_2$, $MgCl_2$, electron donors and inhibitors of PS II

An interesting interrelation between TL_{-55} and TL_{-30} is also revealed when $MnCl_2$ is added to the completely extracted DT-20 subchloroplast particles (Figs. 4B and C). Addition of $MnCl_2$ at a final concentration 2 μM (corresponding to nearly 4 Mn atoms per reaction center of PS II) leads to some activation of the bands peaking at $+65$, $+15$ and $+35°C$, but does not restore the glow peaks around 0°C (Fig. 4C). This result is in agreement with the observation that although a high rate of Hill reaction from Mn^{2+} to dichlorophenolindophenol (DCIP) can be measured in the Mn-depleted DT-20 subchloroplast particles, the oxygen evolution of these particles is only partially reactivated upon readdition of Mn^{2+} [17,19]. The restoration of the termoluminescence bands assigned to the S_2 and S_3 states [11,12] cannot be achieved even if, in addition to Mn^{2+} cations, Ca^{2+} is also added to the depleted particles (not shown). On the other hand, the addition of Mn^{2+} induces remarkable changes in the low-temperature glow peaks. Increase of Mn^{2+} concentration from 0.5 to 2 μM leads to a progressive decrease of TL_{-30} accompanied by an increase of TL_{-55}, so that in the presence of 2 μM $MnCl_2$, TL_{-55} dominates in the glow curve and TL_{-30} is practically abolished (Fig. 4). (This circumstance allowed us to make more accurate measurements of dependence of TL_{-55} on T_{ex} (Fig. 3)). Inhibition of TL_{-30} upon addition of $MnCl_2$ to the completely extracted DT-20 subchloroplast particles can also be observed after excitation at -30 or $+5°C$, and the effect is saturated at an $MnCl_2$ concentration of nearly 2 μM (or nearly 4 Mn atoms per reaction center of PS II). At the same Mn/reaction center ratio, restoration of PS II activities (photoreduction of DCIP in the Hill reaction, photoreduction of Q_A and pheophytin, the intermediary electron acceptor of PS II [20]), is observed [17]. Addition of $MgCl_2$ (5 μM) does not induce as dramatic change in the glow curves as does $MnCl_2$ (Fig. 4D).

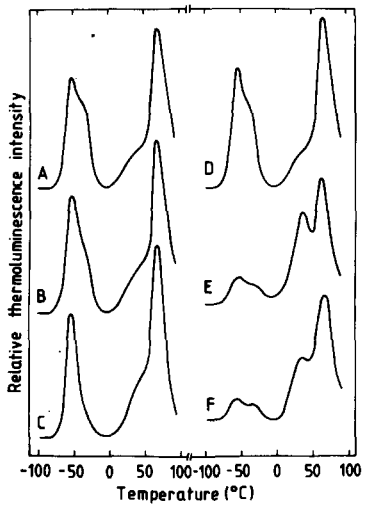

Fig. 4. Effect of different agents on thermoluminescence of the 'completely extracted' subchloroplast PS II particles DT-20, excited by 1 min illumination at $-60°C$ followed by illumination during cooling (40 Cdeg/min) to $-100°C$. A, no addition; B, 0.5 μM $MnCl_2$; C, 2 μM $MnCl_2$; D, 5 μM $MgCl_2$; E, 1.6 μM DCMU; F, 4 min preincubation at $+45°C$.

Addition of DCMU (1.6 μM) leads to a nearly 80% inhibition of the bands of TL_{-30} and TL_{-55} and a 2–3-fold increase of the band around $+35°C$; the $+65°C$ band is slightly diminished (Fig. 4E). (The TL_v excited at $-80°C$ is decreased by a factor of about 2 upon addition of 1.6 μM DCMU.) Similar effects of inhibition of TL_{-30} and TL_{-55} and activation of the band around $+35°C$ are observed after preincubation of the completely extracted DT-20 subchloroplast particles at 45°C for 4 min (Fig. 4F), known to be characteristic of the inactivation of PS II.

High concentrations of the artificial electron donors to PS II (NH_2OH and DPC), added to the completely extracted DT-20 subchloroplast particles, like $MnCl_2$, induce some inhibition of TL_{-30} accompanied by an activation of TL_{-55}, but the effect is much weaker: 100 μM DPC causes nearly

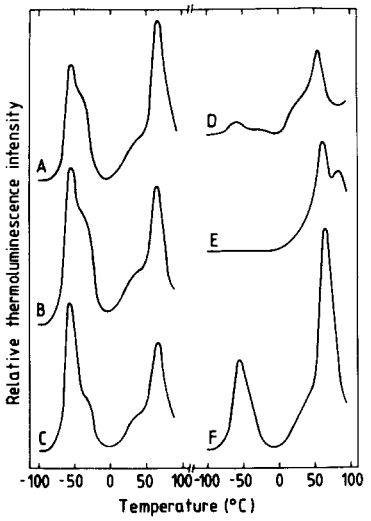

Fig. 5. Effect of various donors of PS II and ferricyanide on the thermoluminescence of 'completely extracted' subchloroplast PSII particles DT-20, excited by 1 min illumination at −60°C followed by illumination during cooling (40 Cdeg/min) to −100°C. A, no addition; B, 20 μM NH_2OH; C, 100 μM DPC; D, 1 mM ascorbate; E, 10 μM dithionite; F, 50 μM ferricyanide.

the same effect as 0.5 μM $MnCl_2$ (Figs. 5B and C). Ascorbate considerably diminishes (Fig. 5D), and dithionite completely eliminates, TL_{-30} and TL_{-55} (Fig. 5E), while the glow peaks at positive temperatures remain unchanged. Ferricyanide does not influence the low temperature bands, but does increase the intensity of the band appearing at +65°C (Fig. 5F).

Identification and origin of the low temperature thermoluminescence bands

The low-temperature thermoluminescence bands of the DT-20 subchloroplast particles (TL_{-30}, TL_{-55}, TL_v) are closely related to charge separation and recombination in PS II reaction centers. This conclusion follows from the effect of both the addition of inhibitor DCMU and thermoinactivation at 45°C which are specific for PS II (Fig. 4).

Properties of TL_{-30} (dependence of its charging on T_{ex}, effect of DCMU and inactivation of the donor side of PS II) are similar to those of the A band described earlier [6,10,16], though the peak position of TL_{-30} is nearly 20 Cdeg lower than that of the A band. In agreement with the results reported in [18,21,22], we consider the TL_{-30} band to be identical to the A band of Inoue [10].

TL_v is identical to the Z_v band, judging from the evident dependence of its peak position on T_{ex} (Fig. 1), which was pointed our earlier for Z_v [6,18,23].

TL_{-55} seems to be a superposition of Z_v with a new (not yet described) band, peaking around −55°C. Thermoluminescence investigations of whole chloroplasts did not lead to the observation of this band [5,6,18,24]. However, in earlier works [18,22] the suspension buffer of chloroplasts contained 0.4 M sorbitol, while in the present investigation the thermoluminescence of the PS II particles was measured in sorbitol-free medium. In order to clarify the actual presence of the TL_{-55} band in the glow curve of chloroplasts and to check simultaneously the possible effect of sorbitol on the TL_{-30} and TL_{-55} bands, we carried out a comparative investigation of the thermoluminescence of chloroplasts in the presence and absence of sorbitol. In Hepes buffer containing 0.4 M

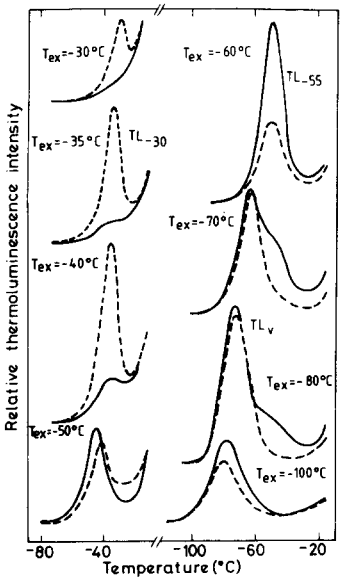

Fig. 6. Dependence of the thermoluminescence intensity of spinach chloroplasts on the temperature of excitation (T_{ex}). Glow curves were excited by 1 min illumination at the indicated temperatures (T_{ex}) followed by illumination during cooling (40 Cdeg/min) to −110°C. Solid line: the suspension medium of chloroplasts contained 50 mM Hepes (pH 7.5) and 35 mM NaCl. Dashed line: the same medium plus 0.4 M sorbitol.

sorbitol, two thermoluminescence bands were observed in the glow curve of chloroplasts between -20 and -100°C: the Z_v band peaking at about -75°C and the A band appearing at about -30°C [18].

Accordingly, in the presence of 0.4 M sorbitol, the Z_v (TL_v) and A (TL_{-30}) bands can clearly be distinguished in the glow curve of spinach chloroplasts (similar results were obtained with pea chloroplasts), but the TL_{-55} band does not appear as a distinct band (Fig. 6, dashed lines). Its contribution to the thermoluminescence can be deduced from the fact that the thermoluminescence intensity remains relatively high between the TL_v and TL_{-30} bands and it does not fall to zero around -55°C. With decreasing sorbitol concentration, the TL_{-30} band gradually decreases, with a concomitant rising of the TL_{-55} band. At 0.3 M sorbital concentration, both the TL_{-30} and TL_{-55} bands can simultaneously be observed in the glow curve (not shown). In sorbitol-free medium, the TL_{-30} band is almost completely abolished and a large TL_{-55} band can be seen when the excitation occurs at -60°C (Fig. 6, solid lines). These results indicate that the TL_{-30} and TL_{-55} bands are interrelated. The TL_{-55} band is intensified at the expense of the TL_{-30} band.

The TL_v (Z_v) band, which exhibits maximum at about -75°C [18], can be observed both in the presence and absence of sorbitol (Fig. 6).

Our comparative experiments demonstrate that under appropriate experimental conditions the same three TL bands, TL_{-30}, TL_{-55} and TL_v, which appear in the glow curve of PS II particles, can also be observed in the glow curve of isolated chloroplasts.

An important conclusion from the data presented here is that the three bands (TL_{-30}, TL_{-55} and TL_v) do not require Mn at all, since they are charged after a complete removal of Mn. The results also indicate that the bands TL_{-30}, TL_{-55} and TL_v arise from charges stabilized on electron carriers which are located closer to the PS II reaction center than the Mn-containing water-oxidizing enzyme. This conclusion is in agreement with the fact that these bands are located at lower temperatures than the B bands, are related to oxygen evolution and disappear upon removal of Mn. Furthermore, TL_{-30} and TL_{-55} could also be observed in preparations of the PS II reaction centers isolated by using detergent Deriphat 160 (not shown). Positive charges related to TL_{-30}, TL_{-55} and TL_v can be located on secondary electron donors of PS II (cytochrome *b*-559, Z, carotene) but additional investigations are needed for clarifying this question.

The data reported here also allows for the conclusion that the traps for charges involved in TL_{-30}, TL_{-55} and TL_v are interrelated so that charging of a thermoluminescence band emitting at higher temperature prevents charging of other thermoluminescence bands appearing at lower temperatures. Evidently, the traps for TL_{-30} and TL_{-55} compete with each other for charges generated by the reaction center. This results in predominant charging of TL_{-30} when $T_{ex} \geqslant -30$°C. At lower T_{ex}, when TL_{-30} cannot be efficiently charged, TL_{-55} becomes charged. This interrelation is especially clearly seen in the experiments on readdition of Mn^{2+} to the completely extracted DT-20 subchloroplast particles. Suppression of TL_{-30} by $MnCl_2$ leads to activation of TL_{-55}. The interrelation of the TL_{-30} and TL_{-55} bands can also be seen in the experiments carried out with whole chloroplasts at various sorbitol concentrations. A decrease in the emission intensity of the TL_{-30} band is accompanied with an intensification of the TL_{-55} band.

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