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Thermoluminescence emission at liquid helium temperatures from photosynthetic apparatus and purified pigments

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Thermoluminescence (TL) emission below 77 K from photosynthetic pigment protein complexes and purified pigments was examined using liquid He. At least four TL components emitting at around 20, 50, 70 and 90 K were resolved on the glow curve from thylakoids. The 20, 50 and 70 K bands are newly observed TL components and designated as Z_α, Z_β and Z_γ bands, respectively. The 90 K band was found to be a different expression of the well-known Z band which was reported as the 110 K band in literatures. These TL bands were evidenced not to be related with charge separation and subsequent electron transfer in reaction centers but originate from light-harvesting chlorophyll (Chl) *a* and *b* by the following observations: (1) red light, which causes charge separation in reaction centers, was ineffective in inducing these TL components; (2) isolated LHCI and LHCII showed higher TL intensities than isolated PS I core and PS II core complexes; and (3) purified Chl *a* and Chl *b* in solid state exhibited essentially the same TL bands. Chl–Chl and Chl–ligand interactions in proteins have been discussed as possible chemical identities of the energy trap responsible for the TL bands.

Introduction

Thermoluminescence (TL) is an emission of light that is observed when irradiated materials are heated. Generally, the emission arises from thermally activated recombination reaction of trapped charges in luminescence centers [1]. TL from plant materials was first reported by Arnold and Sherwood in 1957 [2], who observed dramatically different features of TL from plant leaves depending on the temperature of illumination. Subsequently, more extensive and detailed examinations of the glow curve, i.e., the emission intensity profile against temperature, and its oscillatory behavior under flashing light with isolated thylakoid membranes and pigment protein complexes have revealed that several TL bands emitted from photosynthetic apparatus are due to the photoreaction in Photosystem (PS) II (for a recent review, see Ref. 3).

Between −10 and 30°C, four TL bands have been identified to arise from recombination of charge pairs generated in PS II through light-driven charge separation between the O_2-evolving Mn-cluster as the positive counterpart and the Q_A or Q_B quinone as the negative counterpart. The A band (−10°C) and the Q (or D) band (0–10°C) have been assigned to $S_3Q_A^-$ [4] and $S_2Q_A^-$ [5] charge pairs, respectively. Also, the B_1 and B_2 bands (both around 30°C) have been assigned to $S_3Q_B^-$ and $S_2Q_B^-$ charge pairs, respectively [6]. These TL bands have been extensively utilized to monitor the charge-separated states in PS II, particularly for studying the mechanism of O_2-evolution.

TL bands that are unrelated to the redox-functioning of the O_2-evolving Mn-cluster have also been known. The A_T band appears at around −20°C in Mn-depleted PS II. This band was recently proposed to be associated with a redox-active histidine residue located close to the Mn-cluster [7], although it is not evident whether this histidine residue functions in the water-oxidation process or not. The Zv band that appears between −80 and −30°C depending on illumination temperature is also known to come from PS II, although its origin is still unclear. The Z band that appears at around −160°C [8] is the lowest-temperature band so far observed. Since this band is emitted not only from leaves and thylakoids but also from

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Abbreviations: TL, Thermoluminescence; PS, Photosystem; Chl, chlorophyll; LHC, light-harvesting complex.

boiled leaves [9], isolated chlorophyll (Chl) [9,10] and isolated light-harvesting complex (LHC) I and II [10], it is evidently unrelated with charge separation in reaction centers but is most probably associated with light-harvesting Chl.

Until now all the TL measurements for photosynthetic interest have been performed above liquid N_2 temperature, i.e., 77 K. The TL emission temperature reflects the activation energy of charge recombination or the depth of the trap that stabilizes the charge pairs. If there exists a charge pair having such a small activation energy as can be stabilized only below 77 K, the TL band from this species should appear below 77 K. In this study TL from spinach thylakoids was examined between 6 and 150 K using liquid He. At least four TL bands were resolved in this temperature range, and the origin of these bands were studied by using isolated chlorophyll-protein complexes and purified Chl *a* and *b*.

Materials and Methods

Spinach thylakoids were prepared according to Rutherford et al. [6]. LHCI and PS I core complex were prepared by the method of Haworth et al. [11]. Also, LHCII and PS II core complex were prepared by the method of Burke et al. [12] and Enami et al. [13], respectively. These samples were suspended in 25% (v/v) glycerol, 50 mM Hepes-NaOH (pH 7.0) at a Chl concentration of 2 mg Chl/ml for thylakoids and 0.5 mg Chl/ml for Chl-protein complexes. Chl *a* and Chl *b* were extracted from spinach, purified according to Omata and Murata [14], and then dissolved in CH_2Cl_2 or MeOH at a concentration of 2 mg Chl/ml.

A 70 μl aliquot of the sample solution was spread on a filter paper (1 × 1 cm^2), and cooled with liquid He in a cryostat (Oxford CF1204) equipped with a temperature controller (Oxford ITC4). For measuring purified Chl, two types of preparation were used, i.e., solid state and methanolic solution. For solid Chl, Chl *a* or Chl *b* dissolved in CH_2Cl_2 was charged on a filter paper, and then air-dried for several seconds before frozen. For methanolic solution of Chl *a*, a charged filter paper was quickly frozen before drying.

Samples were illuminated with continuous light from a 150 W tungsten lamp through the cryostat window during lowering the temperature from 200 to 6 K (20–30 min) or at designated temperatures for 10 min. Illumination was performed with white light or colored light passing through a red (Toshiba VR-60, $\lambda > 600$ nm) or blue glass filter (Corning 4-96, $\lambda < 500$ nm).

TL glow curves were recorded by monitoring the emission with a photoncounter (Hamamatsu C-1230) equipped with a cooled red-sensitive photomultiplier (Hamamatsu R550) during heating the sample from 6 to 150 K. Average heating rate was about 15 K/min.

Results and Discussion

Fig. 1 shows a TL glow curve from spinach thylakoids recorded between 6 and 150 K. Among the superposed broad profile appearing in this temperature range, at least four peaks or shoulders are resolved approximately at 20, 50, 70 and 90 K. Although the last two peaks are less resolved and seem to be one broad band around 80 K, it was evidenced that this broad band consists of two different bands (see below). When only the buffer solution applied on a filter paper was measured as a blank experiment, no TL band was observed except for a small peak at around 20 K whose height was less than 1/10 (data not shown) as compared with that of the 20 K band of Fig. 1 glow curve. It is thus clear that the TL bands depicted in Fig. 1 are not artifacts, but originate from thylakoids. Since the sample was continuously illuminated while lowering the sample temperature from 200 to 6 K, the charged species responsible for these TL components were formed and stabilized in thylakoids within this temperature range.

In order to confirm that these TL components originate each from their respective species of charge pairs, light illumination was given at four different temperatures. Fig. 2 shows TL glow curves measured after illumination at 7 K (A), 25 K (B), 50 K (C) and 77 K (D). Under these conditions, the TL components emitting below the illumination temperature will not be stabilized or will quickly decay, so that only the components emitting above the illumination temperature are accumulated to be observed. As clearly shown by the glow curves, the TL bands at 20, 50 and 70 K disappear one by one as the preillumination temperature was raised to 25, 50, 77 K, respectively. This evidences that the four different TL components actually exist in spinach thylakoids at around 20, 50, 70 and 90 K each having different trapping depth.

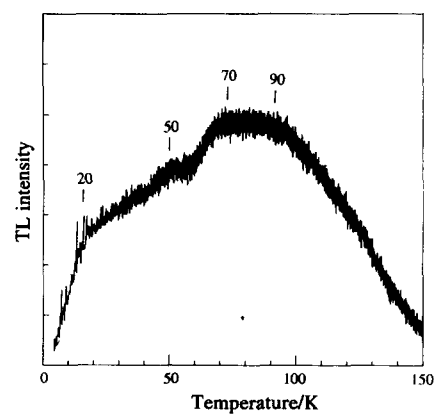

Fig. 1. A TL glow curve from spinach thylakoids recorded between 6 and 150 K. Sample thylakoids were preilluminated with continuous white light while being cooled from 200 to 6 K. Chl concentration of the sample was 2 mg/ml.

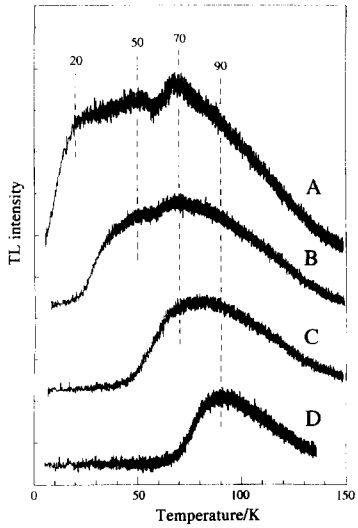

Fig. 2. Dependence on preillumination temperatures of TL glow curves. Sample thylakoids were illuminated with continuous white light for 10 min at 7 K (A), 25 K (B), 50 K (C) and 77 K (D). After illumination, the sample was quickly cooled to 6 K and the glow curve recording was started. Sample concentration was the same as in Fig. 1.

In our previous TL experiment above liquid N_2 temperature, spinach thylakoids preilluminated at 77 K exhibited the so-called Z band at around 110 K (−160°C) [10]. As Fig. 2 shows, however, the glow curve after preillumination at 77 K (curve D) exhibits only one band having maximum at 90 K with no peak or shoulder at 110 K. This implies that the band at 90 K is identical to the Z band, which appeared at 110 K in the previous experiment. This difference in Z-band emission temperature might be attributable to the difference in heating rate between the two TL instruments. In contrast with the 90 K band, the other three bands emitting at 20, 50 and 70 K are detected for the first time in this study. We propose to designate them from now on as Z_α, Z_β and Z_γ bands, respectively.

Fig. 3A shows a TL glow curve of spinach thylakoids preilluminated with red light ($\lambda > 600$ nm) instead of white light during cooling from 200 to 6 K. Only a small peak was observed at 20 K with no other TL bands. As mentioned above, buffer solution on a filter paper also exhibits a similar small band at 20 K (data not shown). Thus the band at 20 K seen on this glow curve does not come from thylakoids but is probably an artifact. The observation that thylakoids do not show any TL bands between 6 and 150 K when preilluminated with red light indicates that none of the Z_α, Z_β, Z_γ and Z bands results from charge separation and subsequent electron transfer in PS I and PS II reaction centers: if the reverse were the case, red light ($\lambda > 600$ nm), which effectively drives the photoreactions in reaction centers, would have contributed to the appearance of the TL bands.

Upon blue-light preillumination ($\lambda < 500$ nm), on the other hand, were observed basically the same band features as observed by white-light preillumination, i.e., the appearance of the Z_α, Z_β, Z_γ and Z bands (Fig. 3B). One possible explanation for the effectiveness of blue light in inducing the TL bands will be ionization of Chl by two-step excitation with higher-energy light: ground-state Chl absorbs blue light and is excited to a singlet excited state. The singlet excited state thus generated will relax to the lowest singlet excited state or to the triplet excited state and finally come back to the ground state. When the Chl molecule in the excited states absorbs another photon before it relaxes to the ground state, the Chl molecule is further excited to a higher-energy electronic level. If this high-energy level exceeds the ionization energy of Chl, and if there are electron donors or acceptors close to the Chl molecule, the Chl molecule releases or accepts an electron to be ionized. This mechanism requires a higher-energy photon that affords ionization of Chl. This might be the reason why red light is ineffective while blue light is effective in inducing these TL bands.

An experimental support for the above assumption can be seen on comparing the glow curve after preillumination with blue light (Fig. 3B) to that with white light (i.e., blue light + red light) (Fig. 1). These glow curves were measured under the same conditions excepting the light quality. Obviously, the emission intensity after white light illumination is higher, particularly at higher temperatures, than the intensity after blue light preillumination. This indicates that red light is effective for the formation of charged species only when blue light is simultaneously provided; the first singlet excited state directly formed by red light absorption or the triplet excited state derived by relax-

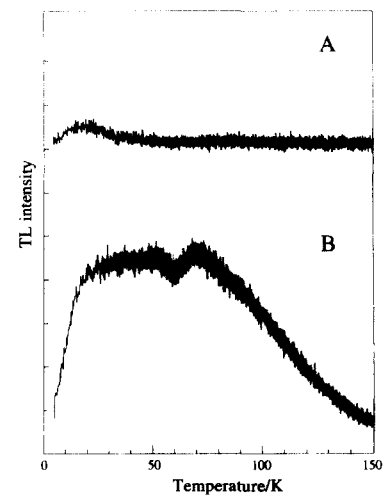

Fig. 3. TL glow curves from thylakoids after preillumination with red light ($\lambda > 600$ nm) (A) or blue light ($\lambda < 500$ nm) (B). The sample thylakoids were continuously preilluminated while being cooled from 200 to 6 K. Sample concentration was the same as in Fig. 1.

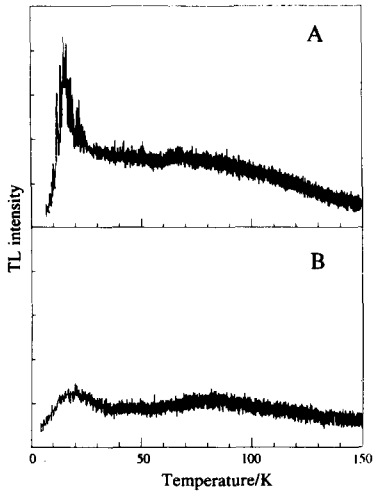

Fig. 4. TL glow curves from isolated LHCI (A) and from isolated PS I core complex (B). Chl concentration of the samples was 0.5 mg/ml. Illumination conditions were the same as in Fig. 1.

ation of this state absorbs again blue light to cause ionization of Chl.

It is obvious from above results that the low temperature TL bands from thylakoids as shown in Fig. 1 are not due to charge separation in reaction centers. To further confirm this, we studied TL glow curves from isolated LHCI, PS I core complex (Fig. 4A and B, respectively), LHCII, and PS II core complex (Fig. 5A and B, respectively). The Chl concentration of these complexes was 1/4 (0.5 mg Chl/ml) as compared with that of thylakoids (2 mg Chl/ml), so that the absolute TL intensities in Figs. 4 and 5 are much lower than those in Fig. 1. In these glow curves, only the 20 K component (the Z_α band) appears well resolved. Although the other bands are not clearly resolved, the broad plateau continuing from the Z_α band up to 150

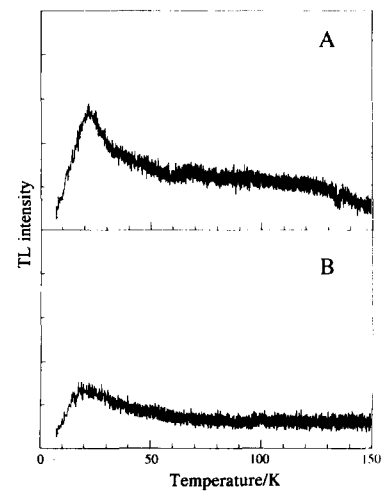

Fig. 5. TL glow curves from isolated LHCII (A) and from isolated PS II core complex (B). Chl concentration of the samples was 0.5 mg/ml. Illumination conditions were the same as in Fig. 1.

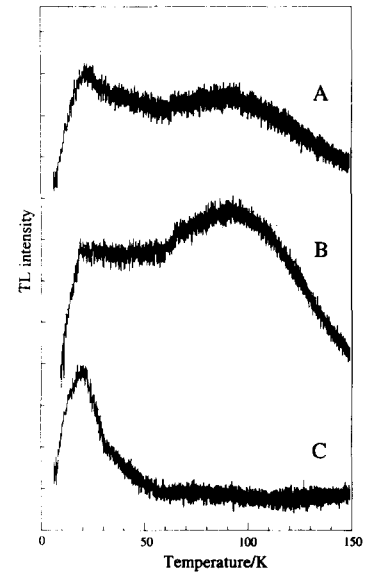

Fig. 6. TL glow curves from solid Chl *a* (A), solid Chl *b* (B), and Chl *a* dissolved in MeOH (C). For solid Chl samples, Chl *a* and *b* dissolved in CH_2Cl_2 (2 mg Chl/ml) were charged on a filter paper and subsequently air-dried for several seconds before cooled. For measurement of Chl *a* in MeOH, MeOH solution of Chl (2 mg Chl/ml) was charged on a filter paper and was quickly frozen before drying. Illumination conditions were the same as in Fig. 1.

K in LHCI, PS I core complex, and LHCII, suggests the existence of the Z_β, Z_γ and Z components in these complexes (Figs. 4A, B and 5A). In PS II core complex, bands other than the Z_α band are very weak or practically absent (Fig. 5B). When the emission intensities from these pigment protein complexes are compared with each other on a Chl basis, LHCI and LHCII always show higher TL emission than their respective core complexes. This hierarchy in the emission intensity of the Z_α-Z_γ bands among the four pigment protein complexes is more or less the same as already observed for the Z band in our previous experiments above liquid N_2 temperature [10]. These results indicate that emission of the Z_α-Z_γ bands does not require reaction centers, but is rather associated with the light-harvesting Chl molecules.

From the above results, purified Chl will be expected to exhibit similar TL bands. Fig. 6 shows the TL glow curves of solid Chl *a* (A), solid Chl *b* (B), and Chl *a* dissolved in MeOH (C). In fact, both Chl *a* and Chl *b* in solid state exhibit a TL band at 20 K accompanied by a broad featureless band around 90 K. On the other hand, Chl *a* in MeOH solution exhibits only a band at 20 K. These data imply that the broad band around 90 K originates from Chl–Chl interactions which are absent in MeOH solution, and the energy for TL emission is presumably stored as a form of Chl^+-Chl^- radical pair. The broad featureless band shape may also suggest that there are many types of Chl–Chl interaction having different trapping depths in the

solid-state Chl prepared in this study. These data may in turn imply that the band at 20 K found in Chl *a* MeOH solution originates from Chl–solvent interaction. Since MeOH is known to form a ligand to the central Mg of a Chl molecule, ligand MeOH will be a plausible candidate counterpart of the Chl radical. The 20 K band, however, appears clearly resolved in both solid Chl *a* and Chl *b* (Fig. 6A and B). This could be due to a ligand formation with water. The solid Chl *a* and *b* samples used in these experiments were prepared by simply spreading the CH_2Cl_2 solution on a filter paper in the air and neither the solvent (CH_2Cl_2) nor the sample (purified Chl) were carefully dried beforehand, so that they may contain small amounts of water which may afford a ligand in place of MeOH. Such ligation of water to Chl has been found in a bacteriochlorophyll *a* film prepared from CH_2Cl_2 solution [15]. We may thus consider that the 20 K band in solid Chl *a* and *b* originates probably from Chl–water interaction.

Taking these results with extracted Chl into account, we may assume the origins of the TL bands emitted from thylakoids below or close to 77 K as follows: the three bands between 50–90 K (Z_β, Z_γ and Z bands) are due to Chl^+-Chl^- radical pairs. Different emission temperatures of these bands may mean the existence of different types of Chl–Chl interactions among the Chl molecules in thylakoids. The possible contribution of carotenoids for the Z band as discussed in our previous paper is unlikely for the Z_α-Z_γ bands, and for the Z band as well, since carotenoid-free Chl *a* or *b* emitted almost the same TL as those from thylakoids. Very probably, carotenoids are not involved in formation and stabilization of the radical pair among Chl molecules. By contrast, the TL component at 20 K (Z_α band) is probably due to a radical pair formed between Chl and a ligand molecule, e.g., an amino acid residue of a protein or a water molecule bound to proteins. In this case, the ligand molecule will act as either an electron acceptor or a donor to Chl.

In conclusion, the TL bands of spinach thylakoids observed at liquid He temperatures do not result from the charge separation driven by the photochemistry in reaction centers. Rather, they appear to arise from radical pairs formed among Chl molecules or between Chl and ligand molecules, although exact identification awaits further studies. The low emission temperatures observed for these bands reflect the small values of activation energy of the recombination in the shallow traps. TL measurements in liquid He temperature range would be a tool to study Chl–Chl and Chl–protein interactions, an aspect of the molecular environments of Chl in photosynthetic apparatus.

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