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Assignment of thermoluminescence A band to $S_3Q_A^-$ charge recombination: sequential stabilization of S_3 and Q_A^- by a two-step illumination at different temperatures

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The origin of thermoluminescence A band emitted from Photosystem II was studied by analyzing the dependence of the charging efficiency of the A band by continuous illumination at −196°C on preillumination flashes and DCMU treatment. The following results were obtained. (1) Illumination at −196°C given after preillumination flashes at 15°C altered the normal oscillation pattern of the flash-induced B band in dark-adapted thylakoids having emission maxima at the first and fifth flashes to a very different pattern showing huge maxima at the second and sixth flashes. (2) The charging efficiency of the A band by the illumination at −196°C oscillated in parallel with the altered oscillation pattern of the B band. (3) Addition of DCMU after preillumination flashes but prior to illumination at −196°C strongly enhanced the A band at the expense of the B band, and the oscillation pattern of the A band thus obtained was almost identical to that of the B band observed in the absence of DCMU. (4) These phenomena were satisfactorily explained by a sequential formation of specified positive and negative charges at the reaction center by flash preillumination and continuous illumination at −196°C, respectively. (5) In $CaCl_2$-washed Photosystem II particles, where the S_3–S_4 transition is blocked by removal of the three extrinsic proteins, the A-band height as a function of the flash number increased up to second flash but stopped oscillating thereafter. Based on these results, it was concluded that the A band of thermoluminescence arises from recombination between the negative charge on the primary quinone acceptor of Photosystem II (Q_A^-), and the positive charge on the S_3 state of the O_2-evolving system.

Introduction

The thermoluminescence from plant materials arises from the recombination of the positive and negative charges stabilized on the donor and acceptor side of illuminated Photosystem II (PS II) reaction centers. The glow curves of isolated thylakoids usually show several luminescence components peaking at different temperatures,

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PQ, plastoquinone pool; PS II, Photosystem II; Q_A and Q_B, primary and secondary quinone acceptors of Photosystem II, respectively; Z, the secondary electron donor of Photosystem II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Mes, 4-morpholineethanesulfonic acid.

each of which originates from different charge pairs. Of these components, the A band is a component which is emitted around −10°C on warming the thylakoids after illumination at low (−5 to −50°C) temperatures with continuous light [1–4]. The involvement of positive charges stabilized on the O_2-evolving enzyme in the emission of this band was clearly demonstrated by the so-called 'A-band enhancing effect' [3,5], in which the charging efficiency of the A band by illumination at −196°C (with continuous light) was remarkably enhanced by a two-flash preillumination given at room temperature prior to freezing the sample.

On the basis of these results, Inoue [3] once proposed that the positive counterpart of the charge pair for the A band would be the 'frozen S_4 state'. However, this interpretation was inconsistent with other observations [6,7]. First, the threshold temperature for $S_3 \rightarrow S_4$ transition is around −20°C [8], so that a −196°C illumination could not create any S_4 state. Second, a thermoluminescence band very similar to the A band can be observed in Tris-washed thylakoids in which the O_2-evolving enzyme no longer exists [6,9,10].

The first problem raised a question as to what the effect of the illumination at −196°C will be under the conditions where no S-state turnover is allowed. This problem was, however, clearly solved by the findings [11,12] that illumination at −196°C inverts the Q_B/Q_B^- ratio (Q_B, secondary quinone acceptor of PS II) in dark-adapted thylakoids. Upon illumination at −196°C, one electron is delivered per reaction center from cytochrome *b*-559 to Q_A, and then to Q_B upon warming above −30°C [13,14]. The oxidized cytochrome *b*-559 itself does not participate in recombination for thermoluminescence emission [11]. Thus, illumination at −196°C must create one extra negative charge on Q_A without advancement of the S-states. By analogy, we consider that the illumination at −196°C after two preflashes causes one electron to be delivered to Q_A without affecting the S_3 state created by the preflashes. This will result in formation of $S_3Q_A^-$ charge pair which leads to the emission of the A band. This communication confirms these predictions.

As to the second problem, a new hypothesis was recently proposed by Demeter et al. [15,16], in which they assumed that the positive charges on S_3, S_2 and Z^+ (Z, the secondary donor of PS II) equilibrate around the emission temperature of the A band. They concluded that the emission of the A band arises from Z^+ and Q_A^- charge pairs. This hypothesis conveniently accounts for the fact that Tris-washed thylakoids still emit the A band (or a band very similar to the A band). However, the hypothesis contains a few assumptions which appear rather inconsistent with our observations. In this communication we discuss those inconsistencies and propose an alternative interpretation for this problem.

Materials and Methods

Thylakoids were prepared from market spinach by standard methods [17], suspended at 4–5 mg Chl/ml in 0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 50 mM Tricine-NaOH (pH 7.8) and stored on ice in the dark for over 2 h before use. O_2-evolving PS II particles were prepared from spinach as reported by Yuasa et al. [18].

Tris-washing was carried out by suspending the thylakoids or PS II particles in 0.8 M Tris-HCl (pH 8.4 at 0°C) under room light, followed by incubation on ice for 30 min. The treated samples were collected by centrifugation, washed once in 0.4 M sucrose/10 mM NaCl/5 mM $MgCl_2$/50 mM Tricine-NaOH (pH 7.8), suspended in the same buffer and stored on ice in the dark until use. $CaCl_2$-washed PS II particles were prepared as described previously [19,24]. The PS II particles were incubated with 1 M $CaCl_2$/0.4 M sucrose/10 mM NaCl/50 mM Mes-NaOH (pH 6.5) for 30 min, spun down, washed twice, and then resuspended in 200 mM NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5). The untreated and washed particles were kept on ice in the dark until use. All the samples for thermoluminescence measurements were diluted with 0.4 M sucrose/10 mM NaCl/40 mM Mes-NaOH (pH 6.5) at a chlorophyll concentration of 0.25 mg Chl/ml just before the measurements.

Thermoluminescence measurements were done as described previously [2] with the sample on a filter paper (2 × 2 cm). In some experiments the samples were put in a thin cuvette arrangement as described in Ref. 11 to enable the addition of

DCMU immediately after flash excitation. The heating rate was 0.4 Cdeg/s. Samples were illuminated with a xenon flash lamp (5 μs, 4 J, white light) or with continuous red light (at least 630 nm, 0.7 mW/cm^2). EPR Signal II was measured with a JEOL spectrometer model JES-FE1GX as described previously [18,20] at a chlorophyll concentration of 3.0–6.0 mg Chl/ml. Cytochrome *b*-559 was determined spectrophotometrically with a Shimadzu UV-3000 spectrophotometer by the procedure of Bendall et al. [20].

Results and Discussion

Assignment of $S_3Q_A^-$ as the charge pair for the A band

Fig. 1A1 shows the oscillations of the B band of well dark-adapted thylakoids used for the present experiments. After the first flash given at room temperature, a high B band was observed at +35°C, after the second flash the B-band height decreased with a slight shift of the peak position toward lower temperature (+30°C), and after the third flash the height decreased much more. The B-band height plotted against the flash number shows a quadruple oscillation with maxima at the first and fifth flashes and a minimum at fourth flash (Fig. 1A2), in agreement with previous reports [11,12]. The B band has been assigned to arise from recombination of $S_2Q_B^-$ and $S_3Q_B^-$ redox pairs [11,12], and the above oscillation pattern could be well predicted by computer simulation assuming the initial ratios of S_0/S_1 and Q_B^-/Q_B dark-adapted as 25%/75% ($S_0Q_B = 12.5\%$, $S_0Q_B^- = 12.5\%$, $S_1Q_B = 62.5\%$, $S_1Q_B^- = 12.5\%$), the probabilities of single hit, double hits and misses as 0.85, 0.05 and 0.1, respectively, and the luminescence ratio of $S_3Q_B^-/S_2Q_B^- = 1.7$ according to Ref. 12 (Fig. 1A2).

Upon additional continuous illumination at −196°C given to the samples that have been preflashed at room temperature, the glow curves depicted in Fig. 1B1 were obtained. The illumination of non-preflashed samples at −196°C slightly charged the B band, but not the A band (B1-0f). If, however, the sample was given two preflashes before freezing, the illumination at −196°C resulted in a high A band (B1-2f). The charging efficiency of the A band in this case is enhanced (A-band-enhancing effect [3,5]). The enhancement was specific for two preflashes since one or three preflashes were much less effective. The illumination at −196°C also affected the oscillation of the B band. Upon illumination of a once-preflashed sample at −196°C, the B band was decreased by about half (compare B1-1f and A1-1f), whereas the band was much increased when a twice-preflashed sample was used (compare B1-2f and A1-2f). Fig. 1B2 shows the plots of the A and B bands as a function of preflash numbers. Upon the illumination at −196°C, the oscillation pattern of the B band is greatly changed and shows remarkably high maxima at the second and sixth flashes (Fig. 1B2, B band) instead of the original pattern with maxima at the first and fifth (Fig. 1A2). The A band shows more or less the same oscillation pattern but with less intensity (Fig. 1B2, A band), indicating that the enhancement in charging efficiency is specifically increased by two or six preflashes.

The glow curves depicted in Fig. 1C1 are those obtained by similar experiments in which DCMU was added to the sample solution immediately after preflash illumination but before the sample had been cooled to −196°C and illuminated with continuous light. The addition of DCMU resulted in a remarkably strong enhancement of the A band for the twice- and thrice-preflashed samples, concomitant with complete disappearance of the B band (C1-2f, C1-3f). Fig. 1C2 depicts the oscillation pattern for the A band obtained in the presence of DCMU added according to this protocol. The pattern shows sharp maxima at the second and sixth flashes, and closely resembles the oscillation pattern of the B band obtained without DCMU (Fig. 1B2) in both the depth of oscillation and the emission intensity.

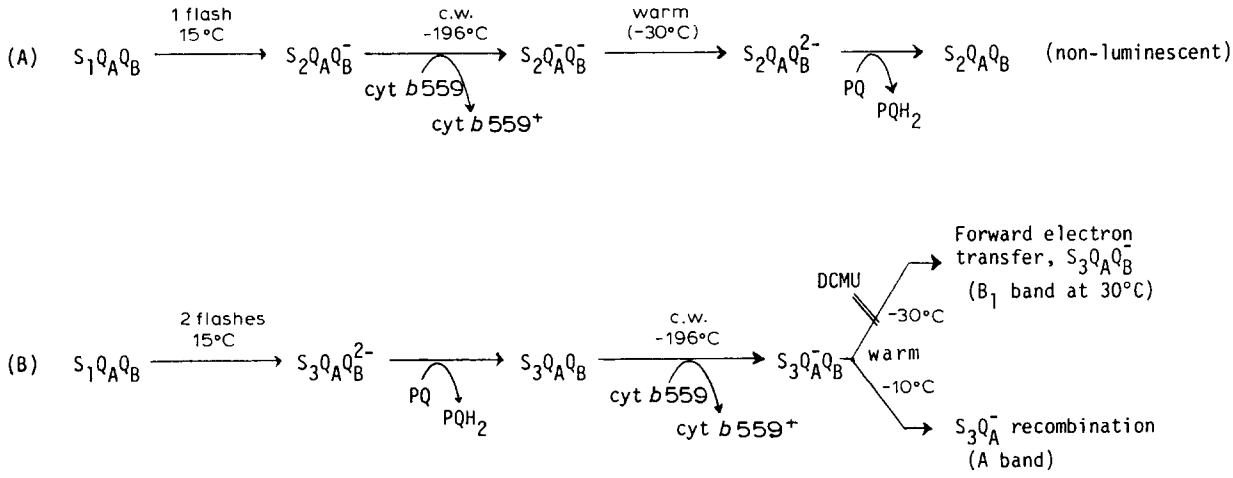
It is noteworthy that the illumination at −196°C in the presence of DCMU after the non- and once-preflashed samples resulted in an emission band at 5–10°C (C1-0f, C1-1f). This band is the so-called D band [2] (or the Q band in Ref. 22), which originates from the recombination of $S_2Q_A^-$ charge pair [11,21]. This charge pair arises from those centers which exist as the $S_1Q_B^-$ redox pair, a minor species in the dark relaxed condition. As we discussed in Ref. 11, 12.5% of the centers are in $S_1Q_AQ_B^-$ state in dark-relaxed

thylakoids. The first flash converts this state to $S_2Q_AQ_B$ (via $S_2Q_AQ_B^{2-}$), which is then converted by illumination at −196°C to $S_2Q_A^-Q_B$ by the electron delivered from cytochrome *b*-559 (regardless of the presence of DCMU). On warming, the $S_2Q_A^-Q_B$ state recombines at 5–10°C as a $S_2Q_A^-$ pair, since forward electron transfer (at −30°C) is blocked by DCMU (see also Scheme I). The same charge pair ($S_2Q_A^-$) can be generated by a single flash of dark-adapted thylakoids in the presence of DCMU, as shown by the broken curve in Fig. 1C1.

These results are satisfactorily interpreted if we assume that the A band arises from the recombination between S_3 and Q_A^-. Scheme I describes the electron transfer anticipated in PS II under the above experimental regime with flash preillumination at room temperature followed by continuous illumination at −196°C. For simplicity, only those centers with the dominant redox pairs ($S_1Q_AQ_B$, 62.5%) in dark-adapted thylakoids are taken into account, and misses and double hits are neglected.

By one preflash (Scheme IA), the centers in $S_1Q_AQ_B$ state are converted to $S_2Q_AQ_B^-$ which emits the B band if the sample is heated. But if these centers are frozen and further illuminated at −196°C, one electron is delivered from cytochrome *b*-559 to Q_A to give $S_2Q_A^-Q_B^-$ [11,12]. Upon warming these centers, the electron on Q_A^- moves forward to Q_B to yield $S_2Q_AQ_B^{2-}$ at around −30°C, the threshold temperature for Q_A to Q_B electron transfer [13,14]. During this warming process, the centers emit neither the A band ($S_3Q_A^-$), because S_3 is absent, nor the D band ($S_2Q_A^-$), because the threshold temperature for $S_2Q_A^-$ recombination is far higher (5–10°C). On further warming, $S_2Q_AQ_B^{2-}$ transfers two electrons to PQ (plastoquinone pool) to yield $S_2Q_AQ_B$. The centers in this state are non-luminescent, since they have no stable negative charges on the acceptor side. This scheme accounts for the reason why the B-band height after illumination at −196°C (Fig. 1B1-1f) is lower than the control (Fig. 1A1-1f). It is of note in this context that the positive charge on cytochrome *b*-559$^+$ and the negative charge on PQ^- do not participate in recombination to emit thermoluminescence [11].

By two preflashes (Scheme IB), on the other hand, the centers in $S_1Q_AQ_B$ state are converted to $S_3Q_AQ_B$ via $S_2Q_AQ_B^-$ and $S_3Q_AQ_B^{2-}$. When the $S_3Q_AQ_B$ centers are illuminated at −196°C, one electron moves from cytochrome *b*-559 to Q_A to yield $S_3Q_A^-Q_B$. Note that no S-state transition accompanies this process. When the centers in this state are warmed, two different processes are expected: (i) above −30°C, the threshold temperature for the forward electron transfer from Q_A to Q_B [13,14], the electron on Q_A migrates to Q_B to yield $S_3Q_AQ_B^-$ state, which on further warming recombines around +30°C to emit the B band



Scheme I. Charge separation and electron transfer expected in $S_1Q_AQ_B$ centers under the excitation regime involving one (A) or two (B) preflashes followed by DCMU addition (only in B) and illumination at −196°C with continuous light (c.w., −196°C).

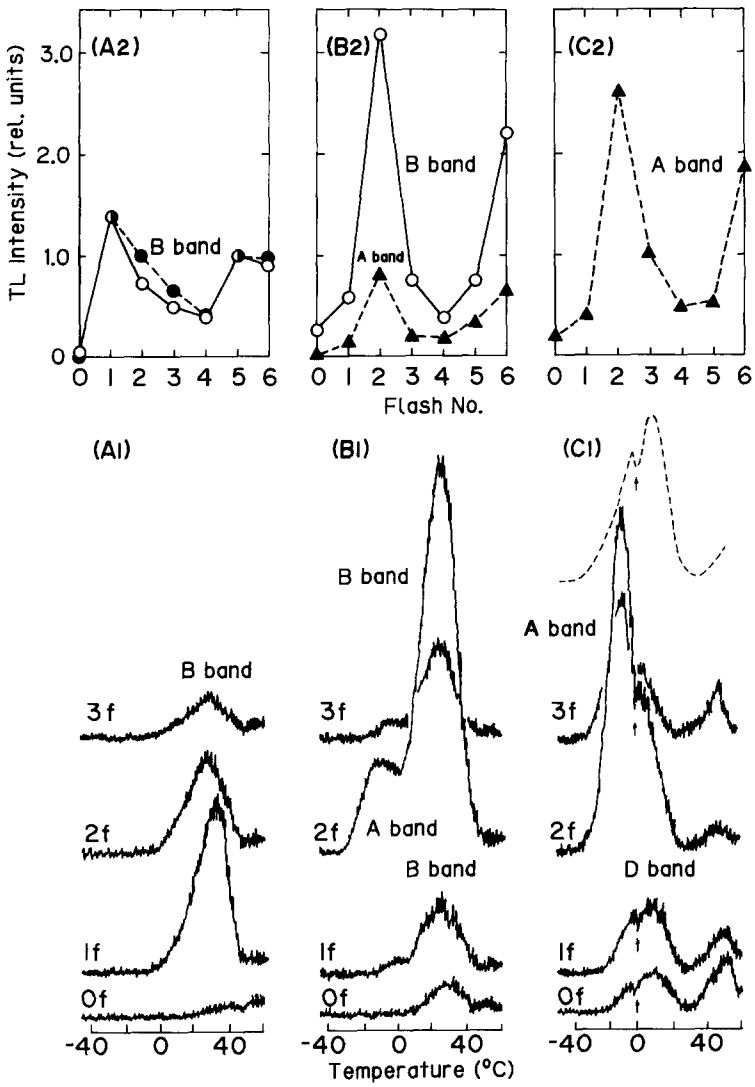


Fig. 1. Oscillation of thermoluminescence A and B bands after three different regimes of excitation of dark-adapted thylakoids: (A) Excitation by indicated numbers (f) of flashes at 15°C; glow-curves (A1) and oscillation pattern (A2) of the B-band height observed (○) and predicted by computer simulation (●). (B) Excitation by illumination at −196°C (continuous light, 1 min) preceded by illumination with flashes at 15°C; glow curves (B1) and the oscillation patterns (B2) of the A band (▲) and B band (○). (C) Excitation by illumination at −196°C in the presence of DCMU (10 μM) added immediately after preflash illumination at 15°C; glow curves (C1) and the oscillation pattern (C2) of the A band (▲). The broken glow curve in C1 show the D band obtained by one flash illumination at 15°C in the presence of DCMU.

(observed as a B_1 band at lower pH values [3,12]); whereas (ii) around −10°C, the electron still remaining on Q_A in some of the centers directly recombines with the positive charge on the S_3 state to emit the A band. These two types of electron transfer compete with each other, since the threshold temperatures of both processes are close to each other. However, because of the slight difference between them, the electrons on Q_A in most of the centers will move to Q_B before the $S_3Q_A^-$ recombination takes place, which results in strong B band at +30°C, while a small remaining portion of the centers undergo $S_3Q_A^-$ recombination to emit a weak A band at −10°C as shown by the glow curve, Fig. 1B1-2f. Note that the luminescence yield for $S_3Q_B^-$ recombination is higher than that for $S_2Q_B^-$ by a factor of 1.7 [12]. If DCMU is added after formation of $S_3Q_AQ_B$ state by two preflashes, the electron on Q_A^- in the $S_3Q_A^-Q_B$ centers supplied from cytochrome *b*-559 by illumination at −196°C will not migrate to Q_B but will remain on Q_A^-, even if the temperature is raised above −30°C, the threshold temperature for $Q_A \rightarrow Q_B$ electron transport. This enables the recombination of $S_3Q_A^-$ in all the centers at −10°C to emit the A band. This accounts well for

the remarkably high A band observed for the twice-preflashed sample with DCMU addition after preflashes (Fig. 1C1-2f). Note that the luminescence yield for $S_3Q_A^-$ recombination is slightly higher than that for $S_2Q_A^-$ by a factor of 1.2 at neutral pH values [23]. It is noteworthy that this interpretation also provides a reason why the B-band oscillation pattern observed in the absence of DCMU (in Fig. 1B2) is identical to the A-band oscillation pattern in the presence of DCMU (Fig. 1C2).

Involvement of cytochrome b-559 as the source of one extra negative charge on Q_A

In the above scheme we assumed that one electron is delivered from cytochrome *b*-559 to Q_A upon illumination at −196°C. This assumption was first introduced by Rutherford et al. [11] to account for the phenomenon that illumination at −196°C inverts the initial Q_B/Q_B^- ratio without affecting the S state. This view was tentatively adopted simply because photooxidation of cytochrome *b*-559 is a well-established photochemical event in PS II at −196°C [13], but no direct evidence has so far been provided for the involvement of this reaction in thermoluminescence processes. In fact, Demeter et al. [14] claim in their recent paper that cytochrome *b*-559 may not be the donor for charging the A band. To attack this problem, we carried out ferricyanide titration of the A band.

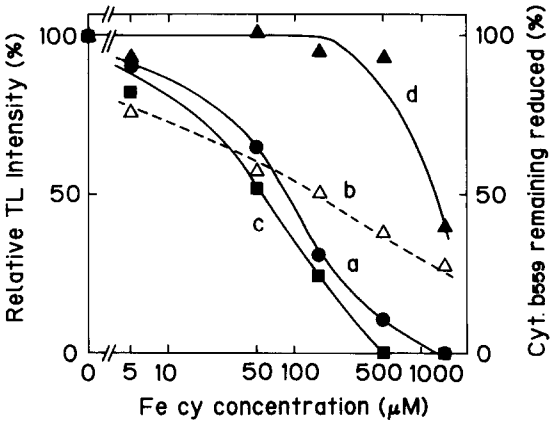


Fig. 2. Ferricyanide titration of thermoluminescence A, B and D bands. Glow curves were measured with the sample thylakoids suspended with varying concentrations of ferricyanide. The A band (●) was excited by illumination at −196°C in the presence of DCMU (10 μM) added immediately after two-flash preillumination at 15°C. The B band (△) and D band (▲) were excited by one-flash illumination at 15°C in the absence and presence of DCMU, respectively. Cytochrome *b*-559 remaining reduced (■) was determined from absorption difference at 559 nm obtained with an excess ferricyanide (2 mM) containing sample as reference.

As shown in Fig. 2, the A-band height obtained with DCMU added after preflashes decreased with increasing ferricyanide concentration and is lost at 1 mM ferricyanide (curve a). This titration curve coincided with the amount of cytochrome *b*-559 remaining reduced (photooxidizable at −196°C) in the presence of varying concentrations of ferricyanide (curve c). The identical titration curves, however, do not directly prove the involvement of cytochrome *b*-559, since ferricyanide will oxidize Q_A^- (the negative charge for the A band) as well as cytochrome *b*-559, and both would result in similar loss of the A band. This possibility was excluded by titration of the B band and D band which arise from $S_2Q_B^-$ and $S_2Q_A^-$ recombinations, respectively. As shown by curves b and d, the B and D bands were more resistant to ferricyanide. Only 60% of the B band and less than 10% of the D band are lost at the ferricyanide concentration (0.5 mM) which completely oxidizes cytochrome *b*-559 (and abolishes the A band). This indicates that cytochrome *b*-559 is much more sensitive to ferricyanide than Q_B^- or Q_A^-, and that the decrease in A-band height is not due to the loss of negative charge by oxidation of Q_A^-, but to the loss of photooxidizable (at −196°C) cytochrome *b*-559. These results strongly support the charging mechanism of the A band indicated by Scheme IB.

A-band oscillation in $CaCl_2$-washed PS II particles

In order to confirm further the involvement of the S_3 state in the recombination for the A band, the A-band oscillation pattern was investigated in $CaCl_2$-washed PS II particles in which the $S_3 \rightarrow S_4$ transition is inhibited. As reported previously [11,12] and shown by the oscillation pattern with open circles in Fig. 3A2, the normal O_2-evolving PS II particles show a typical B-band oscillation after series of flashes [19]. The B band of the $CaCl_2$-washed PS II particles shows normal oscillatory behavior up to the second flash, but the

oscillations are abolished thereafter (solid circles in Fig. 3A2). This pattern agrees with our previous results [19], and analysis of this pattern by computer simulation revealed that $S_3 \rightarrow S_4$ transition is blocked in $CaCl_2$-washed particles [19].

Fig. 3B1 shows the glow curves of $CaCl_2$-washed PS II particles obtained after illumination at −196°C. Similar to the experiments with thylakoids, illumination at −196°C of non- or once-preflashed samples did not charge the A band (B1-0f, B1-1f), whereas illumination of the twice-preflashed sample at −196°C charged a low but appreciable A band (B1-2f). The A-band height in this case did not decrease after the third preflash (B1-3f). When the heights of A and B bands are plotted against the number of preflashes, the oscillation patterns depicted in Fig. 3B2 are obtained. As expected, the oscillatory behavior of both bands is abolished after the second preflash and show a monotonously rising (A band) or declining (B band) pattern. The gradual rising of the A-band height may be due to the contribution by the centers which were in S_0 (25%) state in the initial relaxed condition. Upon DCMU addition after preflashes, the glow curves in Fig. 3C1 are obtained. Although there is an appreciable enhancement in the −5 to 0°C luminescence component accompanied by the disappearance of the B band, the DCMU-induced conversion of the B band to the A band is not as complete as in normal thylakoids (Fig. 1). It is difficult at present to give a reasonable interpretation for this. We speculate that it is partly due to the higher emission temperature of the A band in $CaCl_2$-washed particles based on the following. The shift of the B band to lower temperatures on the second flash (Fig. 3A1-2f) is not as much as in thylakoids (Fig. 1A1-2f); namely, the $S_3Q_B^-$ pair in $CaCl_2$-washed

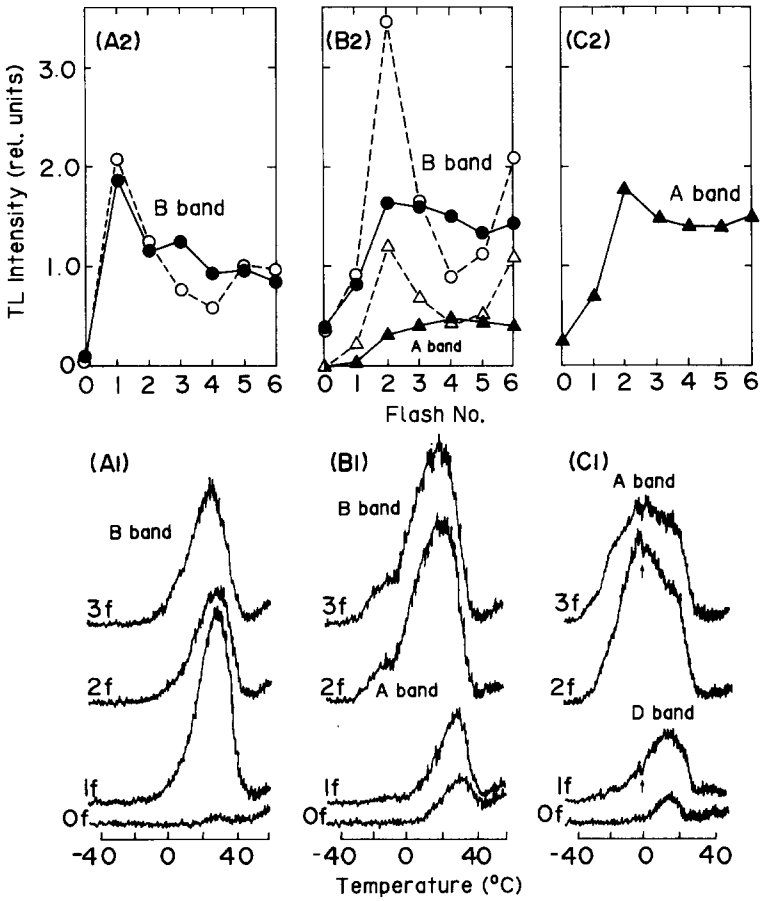


Fig. 3. Oscillation of thermoluminescence A and B bands in $CaCl_2$-washed PS II particles (suspended at pH 6.5) after three different regimes of excitation and treatment as indicated in Fig. 1. In (A1,B1,C1), the glow curves of $CaCl_2$-washed particles are shown, but those of normal particles are not. In (A2,B2,C2), the solid curves are the oscillation patterns of B band (●) and A band (▲) in washed particles, while the broken curves are those of B band (○) and A band (△) in normal particles.

particles recombines at a higher temperature than in normal particles or thylakoids. By analogy, we presume that $S_3Q_A^-$ (A band) in the washed particles recombines at -5 to 0°C rather than at -10°C. When the band heights at -5 to 0°C are plotted, the oscillation pattern depicted in Fig. 3C2 is obtained. This pattern again resembles the B-band oscillation pattern in the absence of DCMU (Fig. 3B2, solid circles). In spite of the ambiguities, these data are well interpreted by the inhibition of $S_3 \rightarrow S_4$ transition in $CaCl_2$-washed PS II particles, and indicate that the positive counterpart of the recombination for the A band is the S_3 state.

However, it is reported that a large part of the high-potential cytochrome *b*-559 is converted to low-potential form after $CaCl_2$ wash [25], which loses the ability to deliver one extra electron to Q_A upon -196°C illumination. In order to clarify this point, we determined the abundance of cytochrome *b*-559 in $CaCl_2$-washed particles (Table I). The untreated particles contained two cytochrome *b*-559 per 240 Chl, one hydroquinone-reducible form (high potential) and the other dithionite-reducible form (low potential). In $CaCl_2$-washed particles, about 80% of cytochrome *b*-559 were found as the ascorbate- or dithionite-reducible form, but a significant amount (0.6 mol/240 Chl) remained still reduced after $CaCl_2$ wash. This amount accounts well for the A band and B band observed after illumination at -196°C of the twice-preflashed sample. Thus, we may consider that some of the centers in $CaCl_2$-washed particles preserve cytochrome *b*-559 capable of delivering one electron to Q_A upon illumination at -196°C. Presumably, the lower A band or the reduced enhancement of the B band in the washed particles as compared with those in normal PS II particles or thylakoids may reflect the limited amount of cytochrome *b*-559 available as electron donor in $CaCl_2$-washed particles.

On the origin of the A band in Tris-washed thylakoids

From the results reported here we conclude that thermoluminescence A band arises from $S_3Q_A^-$ recombination. However, this interpretation disagrees with the observation that a band very similar to the A band can be observed in Tris-washed thylakoids which are depleted of the S states [6,7,9]. When, however, we carefully compare the conditions required for charging of the A bands in the two cases, the following differences can be pointed out (the A band observed in Tris-washed thylakoids is tentatively denoted as the A_T band in the following): Firstly, the A-band intensity oscillates depending on the number of preflashes, while the A_T band does not show any dependence on preflashes (data not shown). Secondly, the A band can be charged by continuous illumination at any low temperatures above -196°C (if preceded by two or more preflashes), while the A_T band can be charged only above -50°C, with maximal efficiency at about -15°C (Fig. 4). Thirdly, in the presence of DCMU the A band cannot be charged by continuous illumination at low temperatures between -50 and -5°C (this condition results in formation of $S_2Q_A^-$ charge pair which gives rise to the D band [11]), while the A_T band can be charged regardless of the presence and absence of DCMU (Fig. 4). This suggests that the negative counterpart of the charge pair for the A_T band is Q_A^- or the reduced form of some other

TABLE I

CONTENT OF CYTOCHROME *b*-559 IN NORMAL AND $CaCl_2$-WASHED PS II PARTICLES

Total refers to 1 mg/ml dithionite minus 1 mM ferricyanide; high potential, 1.5 mM hydroquinone minus 1 mM ferricyanide; low potential, 1 mg/ml dithionite minus 1.5 mM hydroquinone; remaining reduced, no addition minus 1 mM ferricyanide; ascorbate reducible, 2 mM ascorbate minus 1.5 mM hydroquinone.

	Cytochrome *b*-559/240 Chl				
	total	high potential	low potential	remaining reduced	ascorbate reducible
Normal (control)	1.99	0.99	1.00	1.33	0.46
$CaCl_2$-washed	1.93	0.23	1.70	0.60	1.17

acceptor(s) before Q_A. Fourthly, the A band can be charged at a higher efficiency at acidic pH [3], while the charging efficiency of the A_T band sharply decreases at low pH and is practically lost at pH 5.0 (Fig. 4).

In view of these differences in illumination conditions required for charging, the A and A_T bands do not seem to arise from the same charge pairs, although the almost identical emission temperature of the two bands would suggest that both bands arise from the same charge pair. One possible explanation has been proposed by Demeter et al. [15,16]. They assumed that the charge pair for the A_T band is $Z^+Q_A^-$, and that the charge pair for the A band ($S_3Q_A^-$) equilibrates with $Z^+Q_A^-$ around its recombination temperature [15,16]. The charge reservoir for the A band is $S_3Q_A^-$, but the recombination takes place between Q_A^- and Z^+, the same pair as for the A_T band. However, this view does not agree with our parallel measurements of the A_T band and EPR Signal II_f which is known to arise from Z^+. As shown in Fig. 4, the temperature dependence of Signal II_f generation is very different from that of the charging efficiency of the A_T band both in the presence and absence of DCMU. A more demonstrative example is depicted in Fig. 5. In the presence of DCMU, continuous illumination of Tris-washed PS II particles at −15°C induces only a weak Signal II_f, but the same illumination almost maximally

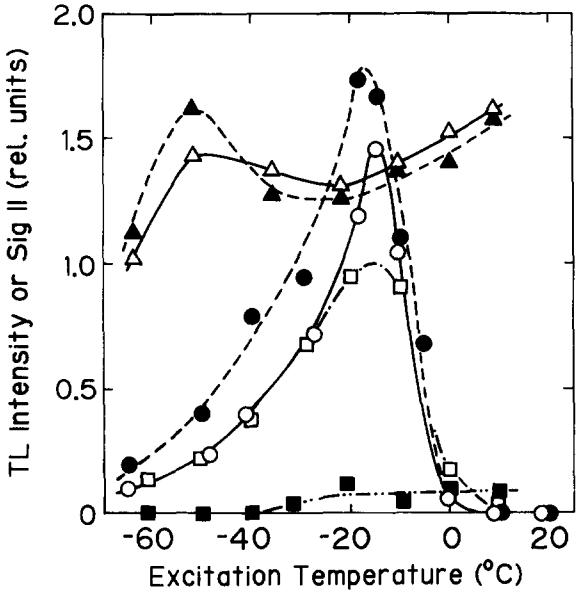


Fig. 4. Temperature dependence of the amplitudes of the A_T band (a band observable in Tris-washed thylakoids having emission characteristics similar to the A band) and EPR Signal II_f. The A_T band was measured with Tris-washed thylakoids which were illuminated at indicated temperatures for 30 s at pH 6.5 in the absence (○) and presence (●) of 10 μM DCMU, or at pH 8.0 (□) and pH 5.0 (■) in the absence of DCMU. Signal II_f was measured at −196°C with concentrated (3 mg Chl/ml) Tris-washed thylakoids which were illuminated at indicated temperatures for 30 s at pH 6.5 in the absence (△) and presence (▲) of 200 μM DCMU and then cooled to −196°C. Instrument settings: modulation amplitude, 4G; time constant, 0.2 s; signal gain, $\times 10^3$; microwave power, 2 mW.

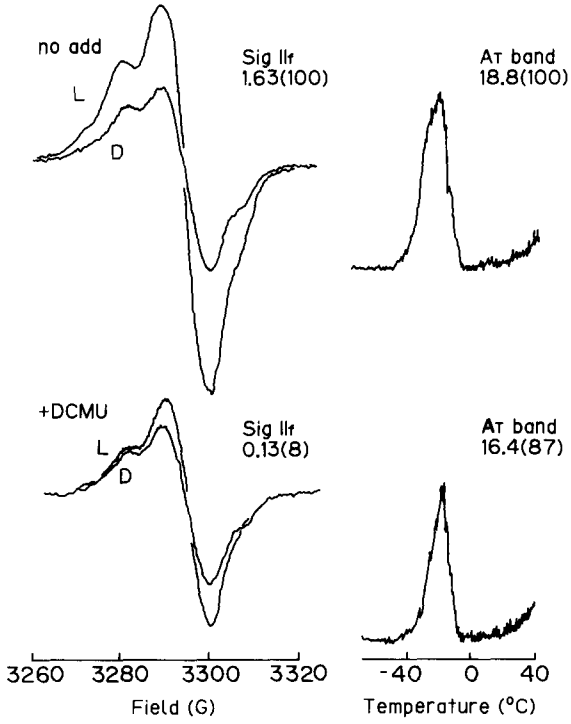


Fig. 5. An obvious discrepancy between the A_T-band amplitude and light-induced Signal II_f amplitude observed under the same illumination condition. EPR spectra of Tris-washed PS II particles measured at −15°C in darkness (D) and during illumination (L) in the absence (upper left) and presence (lower left) of 200 μM DCMU. Light-induced Signal II_f amplitudes were estimated from the light (L) minus dark (D) difference of the peak height at the low magnetic field and indicated with relative values in parentheses. The A_T band was measured with the samples illuminated under the same conditions as for EPR measurements in the absence (upper right) and presence (lower right) of DCMU (10 μM) and the amplitudes are indicated with relative values in parentheses.

charges the A_T band. These results do not provide at present positive information about the charge pair responsible for the A_T band, but at least seem to exclude the possibility that Z^+ is the positive counterpart of the charge pair for the A_T band.

Taking these data and other ambiguities into account, we propose that the A and A_T bands should be considered as different bands, although they share the common negative charge counterpart (Q_A^-). As to the similarities in band shape and emission temperature, we have to speculate at present that they coincide by some fortuitous reasons.

Similar questions must be asked for the A band observable upon continuous illumination of normal thylakoids at -50 to -10°C (the original A band). This charging condition, however, will partly permit the formation of both S_3 and Q_A^-, since the excitation temperature is more or less the same as the threshold temperatures of S_3 formation (-20°C in [8] and -40°C in [16]) and Q_A to Q_B electron transfer (-30°C in [13,14]). Presumably, continuous illumination in this temperature region will directly generate the $S_3Q_A^-$ charge pair in some of the centers (with a certain probability); this is a mechanism different from Scheme IB proposed in this communication. An additional point of concern is the possible superposition of the Z_v band [2,15,16] on this particular A band and on the A_T band, since excitation by continuous light in this temperature range simultaneously charges the Z_v and A bands in both normal and Tris-washed thylakoids.

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