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CHARGING OF THE A BAND OF THERMOLUMINESCENCE, DEPENDENT ON THE S_3 STATE IN ISOLATED CHLOROPLASTS

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Summary

The intensity of the A band of thermoluminescence from isolated spinach chloroplasts was greatly dependent on the excitation temperature between —40 and —10°C. This dependency was analyzed in relation to the S states in the oxygen-evolving system, and the following results were obtained.

1. The A band was charged only slightly by excitation at low temperature (—65° C) with continuous light, but when two flashes had been given before cooling the A band was charged efficiently by the low temperature excitation.
2. The efficiency of the double flash pre-illumination for enhancing the A band showed a steep temperature dependency which agreed with the temperature dependency of the photoconversion from the S_2 to S_3 state.
3. The A-band-enhancing effect was dependent on the number of flashes, and underwent a quadruple oscillation.
4. The enhancing effect decayed in darkness and the decay course agreed with that of the B_1 band but not with that of the B_2 band.

From these results, the origin of positive charges for the A band was discussed.

Introduction

The glow curves of mature green leaves usually show six luminescence peaks emitting at different temperatures [1—3]. These bands were denoted as Z, Z_v, A, B_1, B_2 and C bands [4] according to the nomenclature of Arnold and Sher-

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Abbreviation: PS II, Photosystem II; Tricine, N-tris(hydroxymethyl)methylglycine.

wood [1], with partial modification. Of these bands, the A, B_1 and B_2 bands emitting at around −10, +25 and +40°C, respectively, are closely related to the oxygen-evolving system of chloroplasts [5—7].

It was demonstrated that angiosperm leaves greened under widely spaced flashes or dark-grown gymnosperm leaves, in which the oxygen-evolving system remains latent, do not emit these bands, and that activation of this latent oxygen-evolving system by continuous light or by short interval flashes causes concomitant development of these bands. However, the characteristics of the three bands differ from each other. The B_1 and B_2 bands are efficiently charged by illumination with saturating flashes: the first flash charges the B_2 band and the second flash charges the B_1 band. On the other hand, the A band requires continuous illumination to be charged and its charging efficiency is much lower than those of the B_1 and B_2 bands [8]. Another conspicuous difference between the A and the B_1 and B_2 band lies in the dependence on excitation temperature. The B_1 and B_2 bands were charged efficiently between −65 and +30°C, and were not very dependent on the temperature in this range. By contrast, the A band was charged efficiently when excited between −5 and −20°C but very slightly when excited below −40°C. This strong dependency on excitation temperature is one of the outstanding characteristics of the A band.

Previous studies [8,9] demonstrated that the B band (a composite of the B_1 and B_2 bands) undergoes a quadruple oscillation in intensity when excited with flashes. The oscillation pattern showed maxima at the 2nd and 6th flashes and minima at the 4th and 8th flashes, which is similar to the oscillation pattern of the slow component of delayed emission [10] or triggered luminescence [11]. The oscillation patterns measured at different excitation temperatures between 0 and −65°C suggests that the conversions from S_3 to S_4 and from S_2 to S_3 are blocked at −20 and −35°C, respectively, while the conversion from S_1 to S_2 proceeds below −65°C [9].

Recently we found that the glow curves of the choroplasts suspended in a slightly acidic medium of pH 5.0 show a higher A band than that measured at neutral pH so that the temperature dependence of the A band could be seen more clearly at this acidic pH. The present study was undertaken to clarify the mechanism for charging the A band by using the new experimental system.

Experimental

Chloroplasts were prepared from spinach leaves with 0.05 M Tris-HCl buffer (pH 7.4), 0.4 M sucrose, 0.01 M NaCl, as described previously [8], and suspended in 0.05 M Tricine-NaOH buffer (pH 7.4), 0.4 M sucrose and 0.01 M NaCl at a chlorophyll concentration of about 1.5 mg/ml. The chloroplast suspension layered (2—3 mm in depth) in a Petri-dish was exposed to white light from a 300-W tungsten projector lamp through heat-absorbing filters for 1 min, kept in darkness to be dark-adapted. This pretreatment improved the osillatory behavior of thermoluminescence. An 0.5-ml aliquot of the chloroplast suspension was diluted 10-fold with the Tricine buffer. The pH of the dilution medium was adjusted by NaOH or HCl to be 7.4 or 5.0.

Measurements of glow curves were made as described previously [8]. A 0.1-ml aliquot of the dilute chloroplast suspension (about 15 μg chlorophyll) was spread on a piece of filter paper (2×2 cm^2, Toyo No. 2), cooled to a desired temperature between 0 and −65°C, and illuminated with continuous red light (⩾630 nm) for 15 s at an intensity of about 700 μW/cm^2 (denoted as 'low temperature excitation'). In some experiments, chloroplasts on the filter paper were preilluminated with saturating flashes at a desired temperature before being cooled rapidly to −65°C in liquid nitrogen. The flashes were given from a Xe strobe through an orange filter (⩾560 nm) at intervals of 1 s. The Xe strobe was a kit distributed by U.S. Scientific Instruments, Inc. equipped with a 3CP-6 flash lamp, MCP-3 trigger pack and a capacitor (1 μF) charged by 2.5–3 kV d.c. The duration of each flash was less than 8 μs and the intensity was about 4 J per flash. Immediately after the low temperature excitation, with or without flash pre-illumination, the sample was dipped into liquid nitrogen to be cooled to −77°C. The sample was then heated at a rate of 0.5 K/s up to +80°C, and the luminescence emitted during heating was recorded against temperature, as described previously [6].

Results

1. Dependency of glow curves on excitation temperature and pH

The curves in Fig. 1 show the glow curves of isolated spinach chloroplasts suspended at two different pH values of 5.0 and 7.4 and excited for measurement by 15-s continuous illumination at three different temperatures of T_{ex} = −65, −40 and −20°C. The glow curves depended not only on excitation temperature [4] but also on pH. The chloroplasts suspended at pH 7.4 and excited at T_{ex} = −65°C showed a strong B_2 band at +35°C with an obscure B_1 shoulder around +20°C and weak Z_v and A bands at −50 and −10°C, respectively. The same chloroplast preparation when excited at T_{ex} = −40°C showed a stronger B_2 band with a B_1 shoulder. The emission temperature of the Z_v band shifted to a higher temperature of −30°C, and the A band appeared at −10°C as a small peak filling the trough between the Z_v and B_1 bands. The change of emission temperature of the Z_v band by a change of excitation temperature was reported previously [4,12]. At an even higher excitation temperature of T_{ex} = −20°C, the Z_v band shifted further to overlap on the A band. The composite (Z_v + A) band found around −10°C was about twice as high as these bands observed separately at T_{ex} = −65°C. The B_1 shoulder is masked with the strong B_2 band at this high excitation temperature.

These glow curves of isolated chloroplasts at neutral pH differ considerably from those of intact leaves [5,6]. For example, the A band was usually higher than the B_1 and B_2 bands of intact leaves excited at higher temperatures, while the A band of isolated chloroplasts at a neutral pH could never be higher than the (B_1 + B_2) band. The chloroplasts at the acidic pH of 5.0 were similar in the following respects to intact leaves. The composite B band splits into separate B_1 and B_2 bands. The height of the B_2 band was almost constant, being independent of the excitation temperature, whereas the height of the B_1 band was dependent on the excitation temperature, being charged more efficiently at higher excitation temperatures. The A or the (Z_v + A) band at pH 5.0 was much lower than the B_2 band when excited at T_{ex} = −65°C or −40°C, but was 1.5 times higher than the B_2 band when excited at T_{ex} = −20°C. The A-band

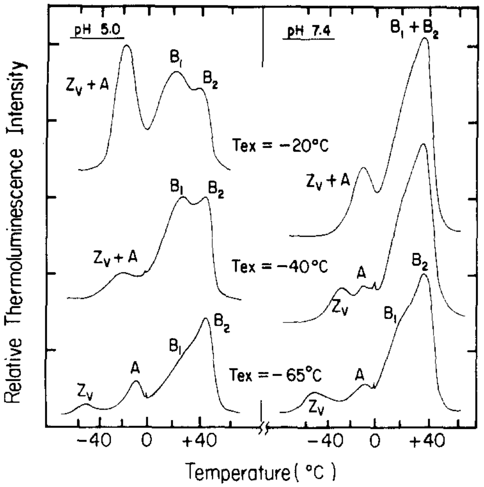


Fig. 1. Glow curves of isolated spinach chloroplasts in neutral (right, pH 7.4) and acidic (left, pH 5.0) suspension, excited by continuous red light for 15 s at three different temperatures of T_{ex} = —20, —40 and —65° C for the upper, middle and bottom glow curves, respectively. The blips between A and B_1 bands are the artifacts due to melting of ice at 0° C.

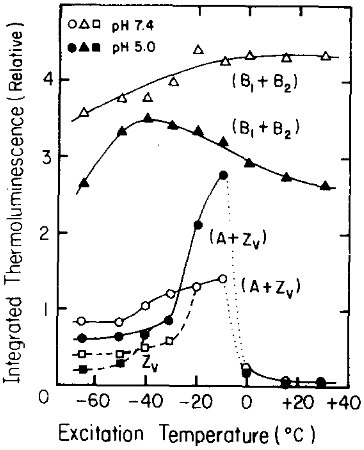


Fig. 2. Dependence of the total luminescence of (Z_V + A) and (B_1 + B_2) bands on excitation temperature at two different pH values. Chloroplasts were excited at various temperatures and the area of each band was integrated against time. Solid and open symbols are the data for the chloroplasts at pH 5.0 and pH 7.4, respectively, and the triangles circles and squares indicate those for the (B_1 + B_2), (Z_V + A) and Z_V bands, respectively.

height showed such marked dependency on the excitation temperature above T_{ex} = —40°C in contrast with the dependency of the B_1 and B_2 bands. A similar distinct enhancement of the A or (Z_v + A) band by raising the excitation temperature has been found for intact leaves [2,4]. The differences in glow curves between intact leaves and isolated chloroplasts seem to be partly due to the pH effect. The pH value in leaf tissues during thermoluminescence measurement could be as low as 5.0 because of the acidic compounds which may leak out of vacuoles due to the effects of freezing and thawing.

Demeter et al. [13] observed an effect of low pH on their +20°C band (B_1 band in this paper) and ascribed it to protonation of plastoquinones on the reducing side of PS II. On the oxidizing side of PS II, positive charges will be more stabilized at low pH than at neutral pH (cf. Fig. 7). Despite these clear effects of low pH, however, the mechanism is not clear as yet.

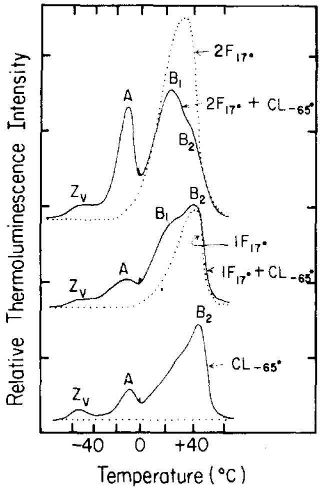
The above marked differences in temperature dependency are more clearly shown in Fig. 2, in which the total luminescence of each band estimated from band area is plotted as a function of excitation temperature. At pH 7.4, the total luminescence of the (B_1 + B_2) band (open triangles) increased gradually with raising of the excitation temperature from —65 to 0° C, and was nearly constant above 0°C. At pH 5.0, the total luminescence of the (B_1 + B_2) band (solid triangles) showed a broad peak around —40°C, decreasing gradually at higher temperatures and rather steeply at lower temperature. Since the B_1 band was not charged efficiently below —40°C at pH 5.0 (cf. the glow curves on the left side of Fig. 1), this steep decrease was mostly due to the lowering of the B_1 band.

The effect of pH was more remarkable on the A and Z_v bands, especially at pH 5.0. The A and Z_v bands were observed separately when excited below −40°C. However, when excited between −30 and −10°C, these two bands merged with each other to give a high (Z_v + A) band; the contribution by the Z_v band to the composite (Z_v + A) band is not appreciable, as will be discussed in the experiment shown in Fig. 3. The (Z_v + A) luminescence (solid circles) rose steeply between −30 and −10°C to show a maximum around −10°C and then dropped nearly to zero above 0°C. This abrupt drop is due to discharge of the positive and negative charges before stabilization at the excitation temperatures close to the emission temperature.

2. *Effect of flash pre-illumination on the dependency of glow curves on excitation temperature*

Fig. 3 shows the effect of flash preillumination on the glow curve of isolated chloroplasts in suspension at pH 5.0. In the experiment, the chloroplasts at +17°C were preilluminated with one or two saturating flashes, cooled rapidly to −65°C, and then illuminated for excitation with continuous red light for 15 s. This 15-s illumination at −65°C is designated as 'low temperature excitation' in further descriptions. The solid curves in Fig. 3 indicate that the pre-illumination with two consecutive flashes with a dark interval of 1 s was highly effective in enhancing the A band, while the pre-illumination with a single flash was almost ineffective. One should note that the low temperature excitation without pre-illumination yielded only a weak A band (bottom glow curve). It is evident from these data that there exists enough capacity for a pool to be oxidized for the A band, but the channel for charging this pool is closed unless two consecutive flashes have been given beforehand. In other words, the A band can be charged efficiently even at −65°C if the oxygen-evolving system is in the S_3 state to which the dark-adapted S_1 state is converted by two consecutive flashes before being cooled to −65°C. It was found that continuous light was necessary for this low temperature excitation. The excitation by a flash at low temperature subsequent to the double flash pre-illumination before freezing could not charge the A band efficiently; more than 10 flashes were needed to have an appreciable height of A band. This seems to be due to the effect of low temperature reported previously [8,9]; at −65°C, only the first flash could charge the B_2 band at a high yield, converting S_1 to S_2, but the further conversion of S_2 to S_3 required several flashes to be completed at low temperature. The failure to observe an intense A band at excitation temperatures below −30°C in the experiments shown in Figs. 1 and 2 may be attributed to incomplete conversion from S_1 to S_3 at the low temperatures.

The dotted curves in the same figure indicate the glow curves measured after the above flash illumination, but without subsequent low temperature excitation commonly applied. A single flash yielded the middle dotted curve, which clearly shows the B_2 band at +40°C, but practically no indication of the B_1 band except the tailing toward lower temperatures. Two consecutive flashes yielded a strong band around +35°C (the upper dotted curve), which seems to be a composite of the B_1 and B_2 bands. It seems evident from these curves that the first flash charged the B_2 band and the second flash charged the B_1 band. The Z_v and A bands were not charged at all by this high temperature excitation. It



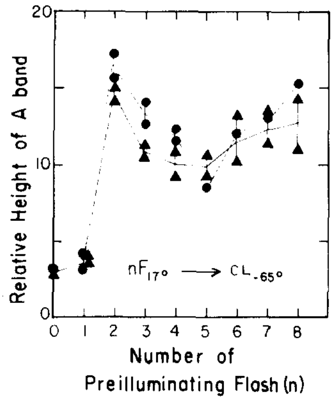


Fig. 3. Effect of flash pre-illumination on the A band height obtained by the post-low-temperature excitation. Chloroplasts (pH 5.0) were illuminated with single (middle solid curve) and double flashes (upper solid curve) at +17°C before being cooled down to —65°C to be provided with the low temperature excitation (CL_{-65°). The bottom solid curve is the reference glow curve measured after the low temperature excitation with no flash pre-illumination. Dotted curves are the glow curves measured after the single (middle) or double (upper) flash pre-illumination without the post-low-temperature excitation.

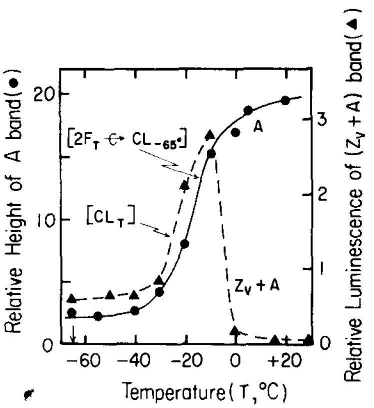
Fig. 4. Effect of the number of pre-illuminating flashes on the enhancement of the A band. Chloroplasts (pH 5.0) were illuminated with different number of flashes at +17°C (nF_{17°), then cooled rapidly to —65°C to be provided with the low temperature excitation (CL_{-65°). The A band height obtained after the low temperature excitation was plotted against the number of pre-illuminating flashes. Triangles and circles indicate the data of two different experiments.

should be noted that the glow curve obtained by the low temperature excitation after double flash pre-illumination (upper solid curve in Fig. 3) shows the Z_v and A bands separately at different emission temperatures of —55 and —10°C, respectively, while the glow curve obtained by excitation at the higher temperature of —20°C shows a composite (Z_v + A) band (upper left curve in Fig. 1). These data indicate that the steep change of the composite (Z_v + A) luminescence dependent on excitation temperature shown in Fig. 2 is mostly due to the change of the A-band intensity.

The above view that double flash pre-illumination is exclusively effective for the A band enhancement was confirmed by the next experiment. Chloroplasts suspended at pH 5.0 were pre-illuminated with different numbers of flashes and excited as usual at the low temperature of —65°C. The A band heights measured as a function of flash number in two separate experiments are shown by solid and broken curves in Fig. 4, which indicate a maximum at the 2nd flash and a minimum at the 5th flash. The maximum at the 2nd flash suggests that the S_3 state is specifically responsible for the A band to be charged by the follow-up low temperature excitation.

3. *Temperature sensitivity of the pre-illumination effect*

Fig. 5 shows the temperature dependency of the A band enhancement by the double flash pre-illumination. As suggested in the previous paper [9], each



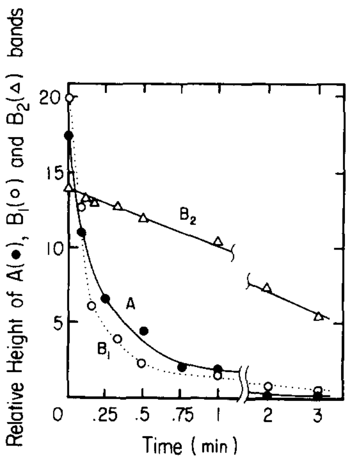


Fig. 5. Dependence of the A band enhancement effect on the temperature of flash pre-illumination. Chloroplasts (pH 5.0) were cooled in darkness to various temperatures (T), illuminated with double flashes, cooled further to −65°C, and then provided with the low temperature excitation ($2F_T \rightarrow CL_{-65°}$). The solid curve A shows the A band height obtained after the low temperature excitation plotted against the temperature of flash pre-illumination, and the broken curve shows the height of the (Z_V + A) band obtained by the same low temperature excitation at various temperatures without double flash pre-illumination (CL_T).

Fig. 6. Decay courses of the A band enhancement effect and B_1 and B_2 band heights during dark incubation after flash pre-illumination. Chloroplasts (pH 5.0) were illuminated with double flashes at +17°C, incubated in darkness for various periods before being cooled to −65°C to be provided with the low temperature excitation. Curves B_1 and B_2 show the heights of the B_1 and B_2 bands, respectively, after the flash pre-illumination, and curve A shows the height of the A band charged by the post-low-temperature excitation.

step of S-state conversion possesses a different temperature sensitivity; the conversion of S_2 to S_3 is blocked at −35°C while the conversion of S_1 to S_2 proceeds at −65°C. In order to examine the effect of pre-illumination temperature, chloroplasts were first cooled to a temperature higher than −65°C, pre-illuminated with two flashes, and then cooled down to −65°C to be excited with 15-s continuous light. The solid curve in Fig. 5 shows the A band height plotted against the pre-illumination temperature. As expected, the A band height depended strongly on the pre-illumination temperature between −30 and 0°C. The double flashes provided below −40°C caused practically no enhancement. The band height increased steeply between −20 and −10°C, and reached saturation at +20°C. It should be noted that the curve drops steeply between −20 and −40°C, which is the critical temperature range where the oscillation pattern of thermoluminescence underwent deformation. The deformation was interpreted previously as being due to the low temperature effect on the S-state photoconversion [9]. The temperature dependency observed here may be ascribed also to the blocking of the photoconversion from S_2 to S_3. The broken line with triangles shows the A band height obtained by 15-s excitation at respective temperatures without pre-illumination. This curve runs parallel with the solid curve up to −10°C, and dropped abruptly because of failure in trapping (cf. Fig. 2). It is evident from these data that the A band can be charged without this failure by illumination of the frozen S_3 state.

4. Decay of pre-illumination effect during dark incubation

Fig. 6 shows the decay of the preillumination effect during dark incubation. After various periods of dark incubation subsequent to the double flash pre-illumination, the chloroplasts were cooled down to −65°C to receive the low temperature excitation. Curve A with solid circles shows the A band height plotted against the illumination time. The curve decreased first rapidly and then slowly to reach almost zero after 1 min. The decay half-time for this first rapid phase was about 0.2 min. Open circles and triangles show the results obtained for the B_1 and B_2 bands charged by double flashes at room temperature without subsequent low temperature illumination. The B_1 band decayed rapidly with a decay half-time of about 0.2 min, whereas the B_2 band decayed very slowly with a decay half-time longer than 1 min. Because of this large difference in the decay time, the glow curves obtained after incubation for more than 2 min showed only the B_2, whereas the curves measured immediately after excitation without dark incubation showed a composite (B_1 + B_2) band. It is clear from these data that the decay of the preillumination effect proceeds almost in parallel with the decay of the B_1 band (i.e. the S_3 state), but not with the decay of the B_2 band (i.e. the S_2 state). Evidently, the S_3 state is responsible for the A band being charged by the subsequent low temperature excitation.

Discussion

The results presented in this paper clearly demonstrate that efficient charging of the A band occurs only when the oxygen-evolving system is set on a specific S state. S_3 is the S state responsible for the A band to be charged for the following reasons. (i) The A-band enhancement was maximally induced by double flash pre-illumination (Figs. 3 and 4). (ii) The temperature dependency of the A-band-enhancing effect (Fig. 5) agreed with that of the photoconversion of S_2 to S_3, as deduced previously from the deformation of the oscillation pattern of thermoluminescence by low temperature [9]. (iii) The pre-illuminated state decayed in darkness with a half decay constant which is roughly equal to the decay constant of the S_3 state estimated from the decay of the B_1 band height (Fig. 6). (iv) In fact, the glow curve obtained after the double flash pre-illumination without the follow-up low temperature excitation (dotted $2F_{17°}$ curve in Fig. 3) seems to show a higher B_1 band component in the composite (B_1 + B_2) band than that in the glow curve obtained after the single flash pre-illumination without the low temperature excitation (dotted $1F_{17°}$ curve in Fig. 3), and the A band is charged by the low temperature excitation at the expense of the B_1 band [14]. A similar phenomenon, charging of one band at the expense of another, was observed by Sane et al. [15].

When these characteristics of the A band are considered in view of the above enhancement mechanism, the conditions needed for charging the A band may be summarized as follows: (a) the S_3 state to be formed beforehand, (b) excitation of the S_3 state at a low temperature which is low enough to block oxygen evolution (the conversion of S_4 to S_0). These conditions imply that the A band can be charged on excitation of the S_3 state kept in frozen state, and suggest that the positive charge pool for the A band is the S_4 state. At room temperature, the S_4 state will be readily converted to S_0 for oxygen evolution, so that we will see no S_4 state. At −65°C, however, we may expect to observe the S_4 state, since the conversion of S_4 to S_0 is blocked at this temperature, as suggested previously from deformation of the oscillation pattern by low temperature [9]. This view that the A band originates from the S_4 state is also supported by the previous observations by us [8] and by Sane et al. [15] that the A band (Sane's Peak I) is charged next to the B_1 and B_2 bands (Sane's Peaks III and IV).

The mechanism for charging the A band proposed here seems to account for the steep dependency of the A band height on excitation temperature which was pointed out by Rubin and Venediktov [16] and more clearly demonstrated in the present study. The A band, when excited with continuous light without flash pre-illumination, is charged in the narrow temperature range between 0 and −20°C and not charged at all below −40°C. As shown in Fig. 5, the temperature dependency found with pre-illumination agreed well with that by 15-s continuous illumination with no flash pre-illumination. This implies that the steep dependency of the A band height is a reflection of the dependency of the photoconversion from S_2 to S_3 state. Probably, the low yield of S_3 state at low temperature determines the lower limit of the narrow range, and the higher limit is determined by the trapping (or emission) temperature of the A band. The temperature sensitivities estimated in the present and previous studies are summarized in the legend to Fig. 7.

A similar enhancement effected by flash pre-illumination has been shown for light-induced oxidation of cytochrome *b*-559 at cryogenic temperature. Vermeglio and Mathis [17] and Amesz et al. [18] observed that the amount of cytochrome *b*-559 oxidized by continuous illumination at −55°C depends on the number of pre-illuminating flashes provided before the chloroplasts are frozen, showing a quadruple oscillation pattern with a maximum at 2nd flash and a minimum at 4th flash. They attributed this phenomenon to the competitive photooxidation between natural primary electron donor of Photosystem II and cytochrome *b*-559, a subsidary donor; more efficient oxidation of cytochrome *b*-559 occurs on illumination of frozen S_2 and S_3 states. The behavior of cytochrome *b*-559 is similar to that of the A band, but these are different from each other in that one flash pre-illumination was appreciably effective (about 70% as effective as two flashes) in enhancing cytochrome *b*-559 oxidation but was almost ineffective for the A band enhancement. Because of this distinct difference, we would suspect that the oxidized cytochrome *b*-559 is not the reservoir of the positive charges for the A band.

As for the B_1 and B_2 bands, we reported previously [8,9] that these bands originate from both S_2 and S_3. The experiment under acidic condition in the present study (dotted curves in Fig. 3), however, revealed that the first flash charges the B_2 band whereas the second flash charges both B_1 and B_2. It is therefore evident that the B_2 band originates from the S_2 state and the B_1 band from the S_3 state. This view is consistent with our previous result [9] that the composite (B_1 + B_2) band oscillates with a four-flash cycle with a maximum at the 2nd flash and a minimum at the 4th flash, and is also supported by the difference in the decay course between these two bands (Fig. 6), although the decay half-times estimated from glow peaks were considerably shorter than those determined for S_2 and S_3 states by Forbush et al. [19] and Joliot et al. [20].

Fig. 7 summarizes the above view of the mechanism for the three thermoluminescence bands in relation to the S states in the oxygen-evolving system, together with the possible intermediate status of water molecules associated with the system. It is assumed in this scheme that a specific thermoluminescence band arises from recombination between the positive charges on a specific S state and the negative charges on a non-specific electron acceptor (A) on the acceptor side of the Photosystem-II reaction center. On thermal activation at an emission temperature of the system frozen after illumination, the positive charges on S states will migrate back to the reaction center; i.e. the electron on reaction center chlorophyll (P) migrates to the positive hole on the partially oxidized [S-water] complex through the primary electron donor (D). This will yield P^+, which will readily react with an electron on A^- to yield an excited reaction center chlorophyll (P★) for luminescence. It is assumed that the emission temperature of each band reflects the activation energy for the positive charge on S states to migrate back to the reaction center over the energy barrier between P and the specific S states. The emission temperature may be determined, in principle, by the rate-limiting step of migration of either positive or negative charge to the reaction center, which is difficult to judge. However, when we consider the above mechanism for charging the A band together with the previous results that the $(B_1 + B_2)$ band undergoes a quadruple oscillation, migration of positive charges from the [S-water] complex to the reaction center seems to be the step thermally activated for luminescence.

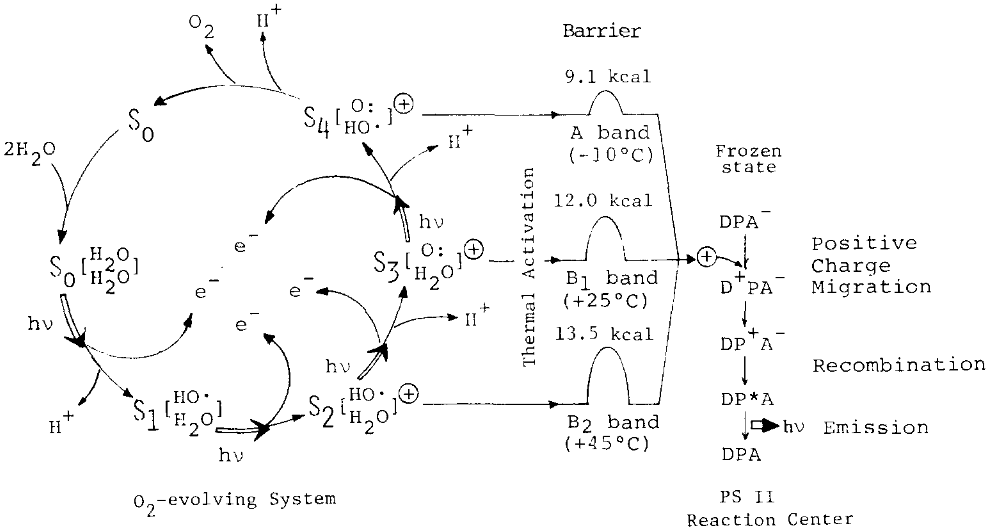


Fig. 7. Possible positive charge traps for the three thermoluminescence bands originating from the oxygen-evolving system. DPA stands for the reaction center of Photosystem II (P) with primary electron donor (D) and acceptor (A), and the Joliot-Kok's S states are shown with the stablized positive charge (+) on the intermediate state of $2H_2O$ molecules postulated according to the H^+ release pattern of 1,0,1,2. Temperature sensitivities of photoconversions of the S states estimated in the present and previous study are as follows: $S_1 \rightarrow S_2$, high yield above $-65°C$ and low yield below $-65°C$; $S_2 \rightarrow S_3$, high yield above $-35°C$ and blocked below $-40°C$; $S_3 \rightarrow S_4$, high yield above $-20°C$ and low yield below $-40°C$.

It is interesting to note in relation to this scheme that, among the intermediate states of the two molecules of water postulated according to the proton release pattern of 1, 0, 1, 2 by Saphon and Crofts [21] and by Fowler [22], positive charges are expected on the three S states, S_2, S_3 and S_4 (as shown by + in Fig. 7), but no positive charge on the S_1 state. Of these three positively charged S states, S_2 and S_3 states are rather easily stabilized by rapid cooling after excitation, since deactivation proceeds gradually at room temperature with a long life time of about 50—90 s [18,19]. By contrast, the S_4 state is labile at room temperature and will readily react with water or its partially oxidized intermediate in a short life time of about 100 μs as determined from the fast component of delayed fluorescence [23], so that the S_4 state will not be stabilized unless the water decomposition has been blocked beforehand by low temperature. These properties of the three S states seem to be well reflected in the characteristics of the three thermoluminescence bands described in the present study.

We suggest that the agreement in number between the three positively charged S states involved in the oxygen clock and the three thermoluminescence bands appearing on activation of the latent oxygen-evolving system may not be fortuitous. Probably, each of the three thermoluminescence bands in question reflects the respective positive charges on the intermediates involved in the water-splitting reaction in photosynthesis.

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