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THE STUDY OF THE PRIMARY PHOTOPROCESSES IN PHOTOSYSTEM I OF CHLOROPLASTS

RECOMBINATION LUMINESCENCE, CHLOROPHYLL TRIPLET STATE AND TRIPLET-TRIPLET ANNIHILATION

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SUMMARY

The dependence of the delayed luminescence of Photosystem I on the state of the reaction centers has been studied. Light flash induces a charge separation in the centers: $P\text{-}700 \cdot P\text{-}430 \overset{h\nu}{\rightleftharpoons} P\text{-}700^{+} \cdot P\text{-}430^{-}$. Dark recombination of charges is accompanied by the recombination luminescence with $\tau_{\frac{1}{2}} \simeq 20$ ms.

If the centers are in the $P\text{-}700 \cdot P\text{-}430^{-}$ state or if *P*-430 is inactivated by heat, then flashing of Photosystem I generates the triplet state chlorophyll with $\tau_{\frac{1}{2}} \simeq 0.5$ ms. The triplet state has been measured by the delayed fluorescence of chlorophyll at 20 °C and 77 °K and by the chlorophyll phosphorescence at 77 °K. The delayed fluorescence at 20 °C arises from the thermal activation of the triplet state up to the excited singlet level of chlorophyll and at 77 °K it is due to triplet-triplet annihilation. The quantum yield of the triplet formation, estimated by a comparison of the light saturation curves of delayed fluorescence at 20 °C and of *P*-700 photooxidation under the same experimental (optical) conditions, is ≈ 0.9 of the $P\text{-}700^{+}$ yield. Only one triplet of chlorophyll can be generated per *P*-700. Under heat inactivation of *P*-430 the triplet formation is not observed when *P*-700 is oxidized.

It is assumed that the triplet-triplet annihilation at 77 °K is related with the strong interaction between the chlorophyll molecules in the pigment complex of Photosystem I. The possibility of a triplet participation in the primary processes of photosynthesis is discussed.

INTRODUCTION

The reaction center of Photosystem I consists of the primary electron donor, chlorophyll *P*-700 [1], and the electron acceptor *P*-430 [2]. The oxidation of *P*-700 is accompanied by the bleaching of the absroption bands at 700, 685 and 430 nm and by the appearance of an EPR signal with $g = 2.0025$ [3]. Reduction of the primary electron acceptor results in the bleaching of a broad absorption band in the blue region at 430 nm [2] and the appearance of an EPR signal of Fe-S centers at

25 °K [4, 5]. It is suggested that the component *P*-430 [6] could be responsible for these Fe-S protein signals. On the basis of the study of recombination luminescence and variable fluorescence under various states of the reaction centers of Photosystem I the interaction between the reaction centers has been suggested [7]. However, the nature of the interaction is not yet clear. The problem concerning the participation of the chlorophyll triplet state in the Photosystem I primary process in chloroplasts is also unsolved [8], although a high quantum yield of triplet formation was found in the bacterial reaction centers [8, 9]. The chlorophyll phosphorescence was found in greening leaves [10].

This paper reports the triplet formation in Photosystem I at 77 °K and 20 °C studied by delayed fluorescence and phosphorescence. The possibility of the center interaction through the triplet level is discussed.

MATERIALS AND METHODS

The "light" fragments of pea chloroplasts, isolated by a modification of the method of Anderson and Boardman [11], were studied. The chloroplasts, suspended in Tris/HCl buffer (pH 8.0, 0.05 M) with 0.5 NaCl (0.5 mg chlorophyll/ml), were treated by 1 % digitonin for 50 min and then by 1 % digitonin and 0.1 % triton X-100 for 10 min. The supernatant after centrifugation at 80 000 $\times g$ for 30 min was recentrifuged at 175 000 $\times g$ for 3.5 h. The pellet (DT-175) was washed twice and resuspended in the Tris buffer with the addition of 10 % glycerol and stored at −196 °C. Before the experiment, the fragments were suspended in 0.05 M Tris/HCl buffer, pH 8.0. To eliminate the residual Photosystem II activity, these fragments were heated at 45 °C for 5 min.

The characteristics of the fragments were as follows: chlorophyll *a*/chlorophyll *b* = 4.95; *P*-700/chlorophyll *a* = 1/150; the rate of 2,6-dichlorophenolindophenol photooxidation in the presence of plastocyanin was 320 μmol/mg chlorophyll per h.

The different absorption spectra ("light minus dark") were measured with a phosphoroscopic setup similar to that described earlier [12]. The dark interval between the actinic and measuring light was 2 ms. It allowed measurement of the absorbance changes without any artifacts, caused by actinic light.

The flash phosphoroscope described earlier [13] was used for the delayed light emission studies. The setup consisted of a 2 μs flash-lamp, a disc phosphoroscope (resolution time, $2 \cdot 10^{-4}$ s), photomultiplier, amplifier, oscilloscope, d.c. amplifier and recorder. The emission spectrum was measured by a grating monochromator.

The flash difference spectrophotometer consisted of the 2 μs flash-lamp, a stabilized measuring-light source, monochromator, photomultiplier and oscilloscope. To observe the flash-induced changes in the region of 400–500 nm, a blue filter (CZC-22) was placed between cuvett (10 × 10 mm) and photomultiplier. Actinic flash was passed through a red filter (KC-14).

RESULTS

Illumination of Photosystem I fragments in the presence of an exogenous electron acceptor or donor results in the accumulation of centers in the $P\text{-}700^{+} \cdot P$-430

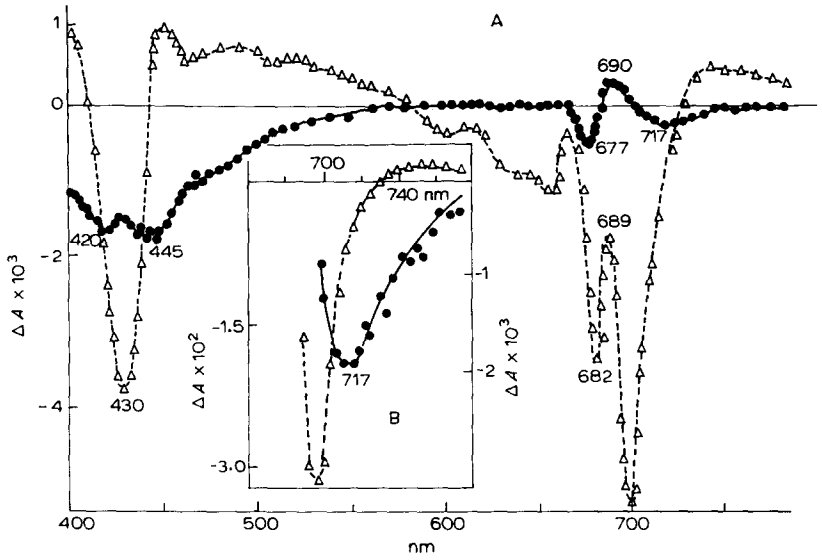

Fig. 1. "Light minus dark" difference spectra of "light" fragments: △–△, in the presence of 25 μM 2,6-dichlorophenolindophenol and 1 mM ascorbate (regime of P-700$^+$ photoaccumulation); ●–●, in the presence of 1 mg/ml dithionite and 10 μM neutral red (regime of P-430$^-$ photoaccumulation). Chlorophyll concentration was: A, 10 μg/ml; B, 70 μg/ml. Actinic light: △–△, 660 $< \lambda <$ 800 nm, $1.8 \cdot 10^5$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1}; ●–●, 700 $< \lambda <$ 800 nm, $0.9 \cdot 10^5$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1}. The data are taken from ref. 7.

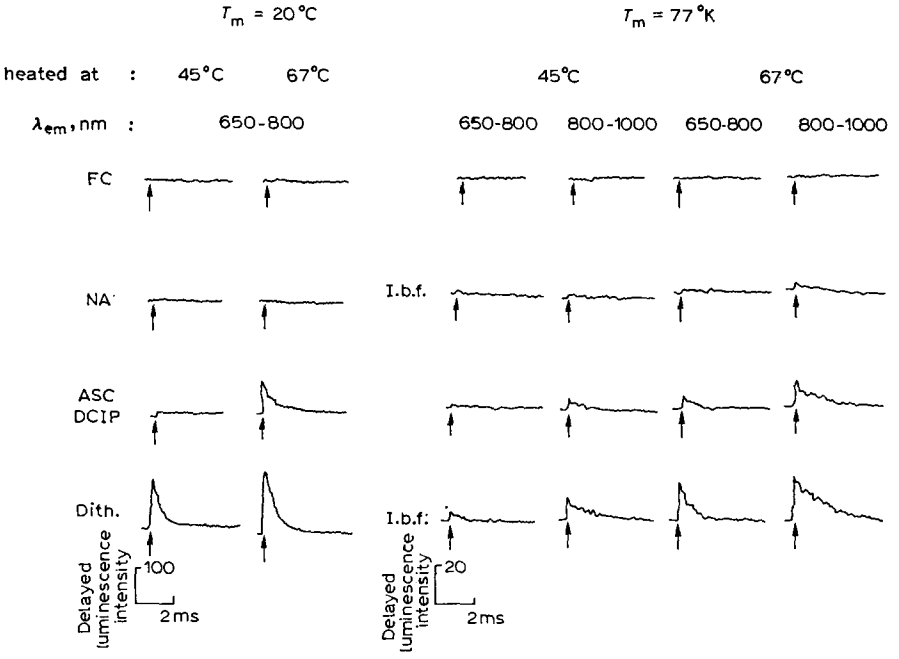

Fig. 2. Delayed luminescence of fragments at 20 °C and 77 °K under vacuum conditions. Exciting flash, > 660 nm, duration 2 μs. Fragments (15 μg chlorophyll/ml) were heated at 45 °C and 67 °C for 5 min. Emission was registered in two spectral regions: 650–800 nm and 800–1000 nm. The reaction mixture contained where indicated: ferricyanide, (FC), 100 μM; sodium ascorbate, 1 mM; 2,6-dichlorophenolindophenol, 5 μM; dithionite, 1 mg/ml; NA, no addition; I.b.f., illumination before freezing.

or P-700 · P-430$^-$ states [6, 7]. Fig. 1 shows the different spectra of P-700 and P-430 obtained under the accumulation regimes of P-700$^+$ and P-430$^-$, respectively. The bands of the P-430 spectrum at 420, 445 and 717 nm are similar to the difference bands of ferredoxin [14]. The longwave shift of the chlorophyll band at 680 nm could indicate either a participation of chlorophyll in the acceptor reaction or an influence of local electrical field of P-430$^-$ on the chlorophyll spectrum.

The flash-induced delayed luminescence of Photosystem I [15] has been

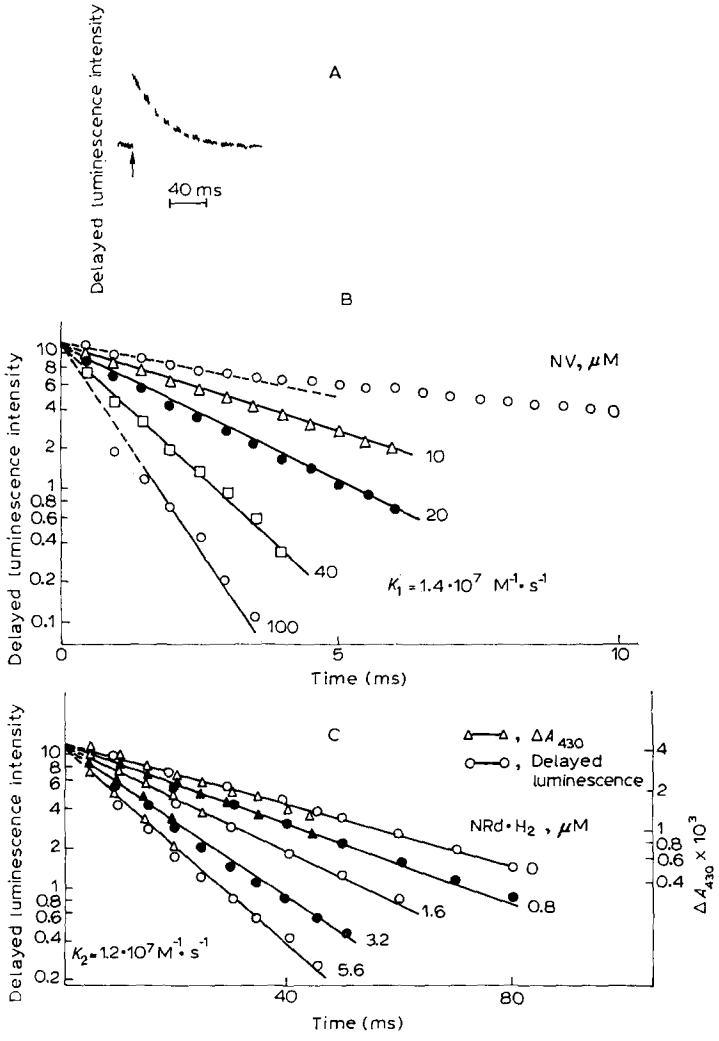

Fig. 3. Decay kinetics of delayed luminescence "slow" component and ΔA-430 (P-700) of fragments after a single flash ($2 \cdot 10^{-6}$ s). Reaction mixture contained: (A) 5 μM 2,6-dichlorophenolindophenol, 1mM ascorbate, 10 μg chlorophyll/ml; (B) various concentrations of methyl viologen (MV), 2,6-dichlorophenolindophenol, 5 μM, 1 mM ascorbate, 10 μg chlorophyll/ml; (C) various concentrations of reduced neutral red (NRd), 1mg dithionite/ml, 10 μg chlorophyll/ml. $K_{1,2}$, bimolecular interaction constants with methyl viologen and reduced neutral red, respectively.

studied depending on the states of *P*-700 and *P*-430. When *P*-700 is present in the oxidized state, obtained by ferricyanide addition or by illumination in the absence of electron donors, no delayed luminescence of fragments is observed (Fig. 2). In the presence of ascorbate and 2,6-dichlorophenoolindophenol, which maintain *P*-700 in an active state in the dark, the flash-induced luminescence with $\tau_{\frac{1}{2}} \simeq 20$ ms is observed (Fig. 3A). The recombination mechanism of this luminescence is supported by the following data. With increasing concentration of an exogenous acceptor (methyl viologen), the rate of luminescence decay became increasingly greater (Fig. 3B), consistent with an increased rate of $P\text{-}430^-$ dark oxidation reported earlier [16]. On the other hand, with increasing concentration of an exogenous donor (reduced neutral red) the acceleration of luminescence decay parallels the increase of $P\text{-}700^+$ reduction rate (Fig. 3C). Thus, the luminescence intensity is dependent on both $P\text{-}700^+$ and $P\text{-}430^-$ concentration, that is, the luminescence probably accompanies the recombination of $P\text{-}700^+$ and $P\text{-}430^-$. The activation energy of the luminescence intensity has been reported to be 0.65 eV [7].

When dithionite is added to fragments under anaerobic conditions (E_h of medium is $\approx -400 + -500$ mV) a new component of the delayed luminescence with $\tau_{\frac{1}{2}} \simeq 0.5$ ms is observed (Fig. 2), the intensity of which is increased reversibly during the illumination (Fig. 4). The increase of the luminescence intensity after a number of flashes parallels the reduction of *P*-430, as measured by its absorption change at 444 nm (Fig. 4). Intensity of this luminescence is 10 times higher than that of the recombination luminescence. The luminescence is observed even in the presence of ascorbate and 2,6-dichlorophenolindophenol after heat inactivation of *P*-430 (Fig. 2). By heat treatment it was found that the appearance of the luminescence parallels the inactivation of *P*-430 and then the inactivation of *P*-700 results in the luminescence decrease (Fig. 5). The luminescence is quenched by oxygen, while in the presence of dithionite it achieves the maximum value.

The maximum of the luminescence spectrum is near 710 nm. The luminescence intensity increases with increasing temperature and the energy of activation is 0.43 eV (Fig. 6).

The luminescence mechanism is further clarified by data on luminescence at 77 °K. The rapid-decaying luminescence at 77 °K as well as 20 °C is observed after the addition of dithionite and illumination of fragments before freezing or after heat

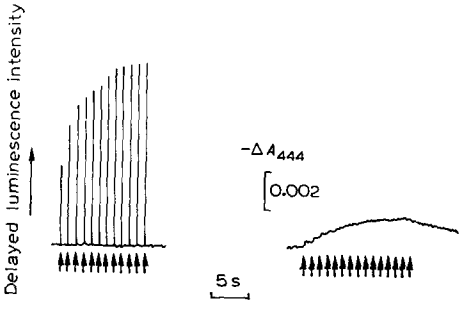

Fig. 4. Dependence of delayed luminescence with $\tau_{\frac{1}{2}} \simeq 0.5$ ms and ΔA-444 (*P*-430) in the presence of dithionite (1 mg/ml) on the number of flashes after the 2-min dark period. Chlorophyll concentration was 15 μg/ml.

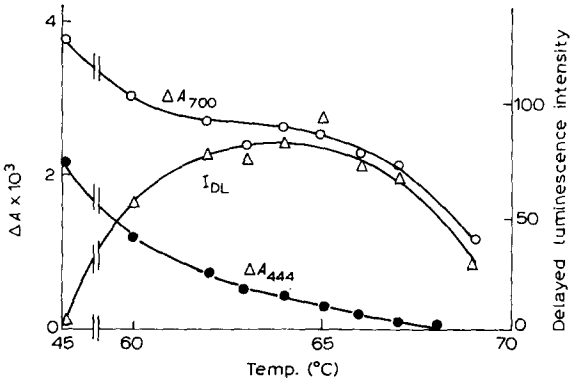

Fig. 5. Heat inactivation of *P*-700, *P*-430 and "fast" component of delayed luminescence with $\tau_{\frac{1}{2}} \simeq 0.5$ ms. Measurement conditions of ΔA-700 (*P*-700) and ΔA-444 (*P*-430) were the same as in Fig. 1. Flash-induced delayed luminescence was measured in the presence of ascorbate (1 mM) and 2,6-dichlorophenolindophenol (5 μM) under vacuum conditions. Chlorophyll concentration was 15 μg/ml. Treatment temperature of fragments (for 5 min) was plotted as abscissa. All measurements were carried out at 20 °C.

inactivation of the fragments (Fig. 2). On the other hand, the luminescence is not observed when *P*-700 is oxidized either by ferricyanide addition or by illumination without any donors before freezing. Maximum luminescence is achieved in the presence of dithionite (Fig. 2).

The luminescence spectrum at 77 °K consists of bands at 960 nm and 740 nm, which correspond to that of chlorophyll phosphorescence [17] and aggregated chlorophyll fluorescence [17], respectively. The two luminescence bands have been studied

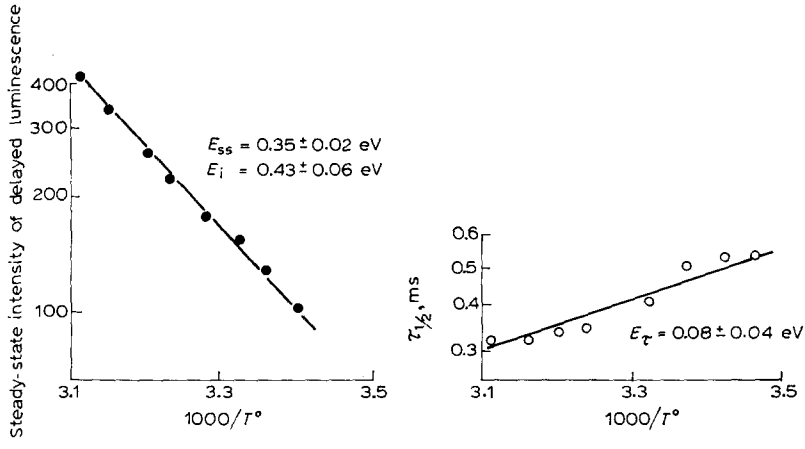

Fig. 6. The temperature dependence of steady-state intensity and lifetime ($\tau_{\frac{1}{2}}$) of delayed luminescence of fragments, heated at 67 °C for 5 min. Delayed luminescence was measured in the presence of ascorbate (1 mM) and 2,6-dichlorophenolindophenol (5 μM) under vacuum conditions. Chlorophyll concentration was 15 μg/ml. Steady-state intensity was registered under continuous illumination ($650 < \lambda < 800$ nm, $I = 10^3$ erg · s^{-1} · cm^{-2}), $\tau_{\frac{1}{2}}$ was measured after exciting flash. E_τ, E_{ss} and E_i are activation energy for lifetime, steady-state and initial intensity of delayed luminescence, respectively.

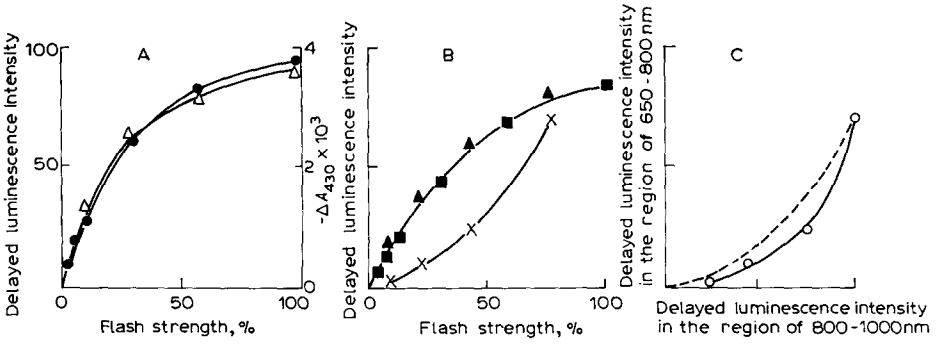

Fig. 7. (A) The light saturation curve of *P*-700, as measured by its absorption changes at 430 nm in the presence of 1 mM ascorbate and 5 μM 2,6-dichlorophenolindophenol (△–△) and also the light saturation curve of the intensity of delayed luminescence (with $\tau_{\frac{1}{2}} \simeq 0.5$ ms), measured in the presence of 1 mg/ml dithionite, 20 °C (●–●). (B and C) The light curves of delayed luminescence intensity in the presence of dithionite (1 mg/ml) of fragments heated at 67 °C for 5 min: ■–■, delayed luminescence at 20 °C in the region of 650–800 nm; ▲–▲, at 77 °K in the region of 800–1000 nm; ×–×, at 77 °K in the region of 650–800 nm; ○–○, the delayed luminescence intensity at 77 °K in the region of 650–800 nm against that in the region of 800–1000 nm. Chlorophyll concentration was 15 μg/ml. Exciting flash, $\lambda > 660$ nm, duration 2 μs.

separately in the 800 nm to 1000 nm and the 650 nm to 800 nm regions. The luminescence at 960 nm has a lifetime of about 1 ms (Fig. 2). The light saturation curve of this luminescence is similar to that of luminescence at 20 °C (Fig. 7B). The band at 740 nm has $\tau_{\frac{1}{2}} \simeq 0.5$ ms (Fig. 2) and the light curve of this band begins slowly and then increases rapidly without saturation (Fig. 7B). The intensity of the 740 nm band approaches a square dependence on the intensity of the 960 nm band (Fig. 7C).

Thus, one can conclude that at 77 °K the band of luminescence near 960 nm is due to chlorophyll phosphorescence and that near 740 nm is caused by triplet-triplet annihilation. At 20 °C the luminescence is due to the thermal activation of the triplet state of chlorophyll up to the excited singlet level of chlorophyll, that is, it is the E-type delayed fluorescence of chlorophyll [18]. The reasons for this conclusion are: the same lifetimes of the two types of luminescence at 20 °C and 77 °K, similar conditions of their appearance and similar light saturation curves, and an agreement of the triplet-singlet transition energy calculated from spectral data (0.45 eV) with that derived from temperature dependence of the delayed fluorescence (0.43 eV) (Fig. 6).

The quantum yield of triplet formation has been estimated by a comparison of the light saturation curves of *P*-700 photooxidation and of the delayed fluorescence at 20 °C under the same optical conditions with 2 μs flash illumination (Fig. 7A). The ratio of the half-saturation intensities of *P*-700 oxidation and delayed fluorescence obtained from exponential dependence of the light saturation curves is about 0.9.

The coincidence of the light saturation curves of delayed fluorescence and *P*-700 photooxidation points to the following conclusions, only one triplet with $\tau_{\frac{1}{2}} \simeq$ 0.5 ms can be generated per *P*-700 and the quantum yield of the triplet formation is about 90 % of the $P\text{-}700^+$ yield. After heat inactivation of fragments at 67 °C for 5 min the quantum yield of the triplet formation is decreased to $\approx$ 60 % of the initial yield.

In whole chloroplasts, cells and leaves the delayed luminescence originates from Photosystem II [19, 20]. The initial lifetime of this luminescence, measured with the high-speed phosphoroscope, is about 10 μs in agreement with [21]. After addition of dithionite to chloroplasts the microsecond luminescence is considerably decreased, which is probably related to the reduction of the primary acceptor of Photosystem II. Under this condition, the chloroplasts exhibit delayed luminescence with $\tau_{\frac{1}{2}} \simeq 0.5$ ms, which is similar to the delayed fluorescence of Photosystem I.

DISCUSSION

Our data show that the dark recombination of the light-induced $P\text{-}700^+$ and $P\text{-}430^-$ is accompanied by the recombination luminescence. The temperature dependence of the luminescence intensity shows that the energy difference between $P\text{-}700^* \cdot P\text{-}430$ and $P\text{-}700^+ \cdot P\text{-}430^-$ states is ≈ 0.65 eV [7]. It corresponds to the energy difference between emitted quanta (≈ 1.7 eV) and the free energy of $P\text{-}700^+ \cdot P\text{-}430^-$ state, estimated from the relationship $G_0 = -nF \cdot \Delta E_m$, were F is the Faraday. As E_m values of P-700 and P-430 are $+0.5$ and -0.55 V, respectively [7], G_0 is ≈ 24 kcal/mol (≈ 1.05 eV).

The contribution of monometric molecules of chlorophyll unconnected with Photosystem I to the delayed fluorescence and phosphorescence of the fragments is possible especially for fragments heated at 67 °C. However, this contribution is probably negligible for the untreated fragments, as suggested by the parallelism between the light saturation curves of the delayed fluorescence and P-700 photooxidation, the increase of delayed fluorescence under illumination at 20 °C and the lack of luminescence at 77 °K when P-700 is oxidized (Fig. 2).

Thus, in analogy with bacterial reaction centers [8, 9], Photosystem I of chloroplasts exhibits the triplet state of chlorophyll when the primary acceptor is reduced. However, the bacterial reaction centers differ from Photosystem I in the lifetime of the triplet states: ≈ 6 μs [8] vs. ~ 500 μs. In the other properties of triplet states there are a number of similarities. Only one triplet of chlorophyll can be generated per P-700. The quantum yield of the triplet formation approaches that of P-700 photooxidation. It is important that under heat inactivation of P-430 and when P-700 is oxidized triplet formation is not observed. It is possible that P-700 itself exhibits the triplet state.

The recombination of $P\text{-}700^+$ and $P\text{-}430^-$ takes place probably through the triplet state of chlorophyll, since the activation energy of recombination (≈ 0.2 eV [7]) corresponds to the energy difference between $P\text{-}430^-$ and the triplet state levels (0.65 eV and 0.43 eV below the level of P-700*, respectively), and also the frequency factor of the recombination ($\approx 10^4\ s^{-1}$) conforms to the rate of the triplet deactivation. The triplet state possibly participates in the primary process of the charge separation, as the increase of the quantum yield of chlorophyll fluorescence under P-700 oxidation or P-430 reduction is only 10 % of the initial value [7], while P-430 reduction results in a considerable increase of the chlorophyll delayed fluorescence and the quantum yield of the triplet formation approaches unity.

The fact of the triplet-triplet annihilation at 77 °K indicates probably a strong interaction between the chlorophyll molecules in the pigment complex of Photosystem I in which the interaction of triplet exciton can take place. Therefore it is possible

that the mechanism of the charge separation in Photosystem I involves the migration of exciton (singlet or triplet) in the pigment complex and its trapping in the mixed levels probably involving Fe. The center interaction, described earlier [7], is probably related to the "solid state" properties of Photosystem I.

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