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# PICOSECOND AND MICROSECOND PULSE LASER STUDIES OF EXCITON QUENCHING AND EXCITON DISTRIBUTION IN SPINACH CHLOROPLASTS AT LOW TEMPERATURES

NICHOLAS E GEACINTOV[a], JACQUES BRETON[b], CHARLES SWENBERG[a], ANTHONY J CAMPILLO[c], RONALD C. HYER[c] and STANLEY L SHAPIRO[c]

[a]*Chemistry Department and Radiation and Solid State Laboratory, New-York University, New York, N Y 10003 (U S A ),* [b]*Département de Biologie, Centre d'Etudes Nucléaires de Saclay, 91190 Gif-sur-Yvette (France) and* [c]*University of California, Los Alamos Scientific Laboratory, Los Alamos N M , 87545 (U S A )*

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## SUMMARY

Studies of the fluorescence quantum yield and decay times, determined at the emission maxima of 685 and 735 nm, using picosecond laser pulses for excitation, indicate that the pigments which are responsible for the 735 nm emission derive their energy by transfer of singlet excitons from the light-harvesting pigments and not by direct absorption of photons. Microsecond pulse laser studies of the fluorescence quantum yields at these two fluorescence wavelengths indicate that long lived quenchers (most probably triplet states), which quench singlet excitons, accumulate preferentially within the long wavelength pigment system which gives rise to the 735 nm emission band.

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The photosynthetic membranes of green alga and higher plants can be fractionated into several different chlorophyll-protein complexes. 10–18 % of the total chlorophyll occurs in a type of protein-chlorophyll complex which is enriched with the *P*-700 reaction centers of Photosystem I [1]. Another 40–60 % of the chlorophyll occurs as protein complexes containing equal amounts of chlorophyll *a* and *b*. These complexes have been designated as light-harvesting chlorophyll-protein complexes [1]. The light-harvesting pigment-protein complexes act as antennae and it has been proposed that they can feed excitation energy to either of the two photosystems A third type of antenna chlorophyll-protein complex is believed to be closely associated with the Photosystem II reaction centers [2]. Based on this tripartite organization of chlorophyll in vivo, Butler and Kitajima have proposed that the low temperature fluorescence bands of chloroplasts at 685, 695 and 735 nm can be assigned to the light-harvesting, Photosystem II antenna and Photosystem I chlorophyll-protein complexes respectively [2, 3] Various fluorescence induction and quenching experiments at 77 K have provided support for this tripartite fluorescence model [3–5]

Recently, laser pulses have been utilized to study the fluorescence of chloro-

phyll in vivo [6–13]. It has been shown that the fluorescence quantum yield decreases with increasing intensity of the laser when single [6, 7] or multiple [12, 13] pulses are utilized for excitation. The fluorescence decay time of chlorophyll in vivo at room temperature also decreases strongly with increasing intensity of the pulse [8] This quenching has been attributed to the mutual annihilation of singlet excitons when single picosecond or nanosecond duration laser pulses are used [8, 14]. When trains of picosecond laser pulses or microsecond-duration intense laser pulses are utilized, the quenching of singlets by relatively long-lived triplet excitons and/or ions can also constitute important quenching channels [6, 8, 12–14].

In this report the quenching efficiencies determined at the 685 and 735 nm emission bands of chloroplasts at low temperatures are compared. While there are striking differences between the quenching curves (plots of relative fluorescence yield as a function of the number of photons per pulse) at 685 and 735 nm when microsecond duration pulses are used, the quenching curves measured at these two emission wavelengths are identical when picosecond duration pulses are employed. In addition, fluorescence decay measurements at these two wavelengths using picosecond laser excitation pulses show that there is a strong variation in the decay times with intensity at 685, but not at 735 nm. These results are interpreted in terms of how the excitation energy is distributed in the photosynthetic units.

The experimental details are described in greater detail elsewhere [8, 12, 13]. The experiments were performed with spinach chloroplast suspensions at 77–100 K. The fluorescence quenching curves were determined at Saclay using a mode-locked dye laser operating at 610 nm. An electro-optic shutter was used to select a single pulse out of the train of about 300 pulses. The microsecond duration pulse was obtained by removing the mode-locking dye from the laser cavity [13]. The fluorescence spectrum (Fig. 1) was determined using a spectrograph-optical multichannel analyzer combination [13]. The fluorescence decay curves were determined with single pulses from a frequency doubled and mode-locked Nd · Yag ($Nd^{+3}$ . Yttrium aluminum garnett)

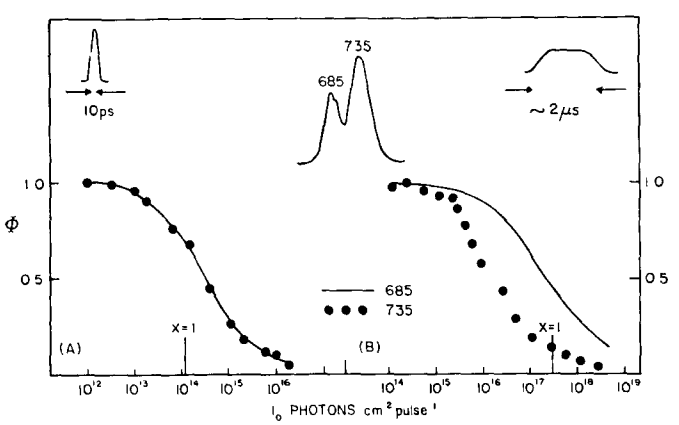


Fig 1 (A) Relative fluorescence yield $\phi$ as a function of the incident intensity $I_0$ of the excitation picosecond laser pulses determined at the emission wavelengths of 685 and 735 nm (spectrum shown in insert) (B) $\phi$ as a function of $I_0$ using a laser pulse of approx 2 $\mu$s duration at 685 and 735 nm All data obtained with spinach chloroplasts at 100 K Excitation wavelength, 610 nm.

laser operating at 530 nm, and a streak camera-optical multichannel analyzer combination [8] at Los Alamos.

The quenching curves for the picosecond and microsecond excitation pulses are compared in Fig. 1. In the picosecond pulse case there is no difference, within experimental error, in the fluorescence yields determined at 685 and at 735 nm. The quenching factor (defined here as the fluorescence yields $\phi$ determined at the lowest intensity, divided by $\phi$ at the highest pulse intensity) is 12 in Fig 1A. With the microsecond pulse, the quenching factors are 25 at 735 nm and only 7 at 685 nm.

On timescales of nanoseconds or less, there is comparatively little time for a substantial buildup of long-lived quenchers of singlets, such as triplet excitons [6, 8, 13, 14] Triplet excitons in the photosynthetic units can act as efficient quenchers of singlets [15]. When a picosecond excitation pulse is employed, it has been proposed [8, 14] that bimolecular singlet-singlet exciton annihilation decreases both the fluorescence yield and the decay times according to the following process:

$$S_1 + S_1 \xrightarrow{\gamma} S_0 + S_n \tag{1}$$

where $\gamma$ is the bimolecular quenching constant, $S_0$, $S_1$ and $S_n$ are respectively the ground state, first and upper (n) excited states of chlorophyll

When a microsecond duration pulse is employed, the number of photons arriving per unit time interval (comparable to the singlet lifetime) per photosynthetic unit is not sufficient to build up a sufficient density of singlet excitons for reaction 1 to occur to a significant extent. Using the molar extinction coefficients for chlorophyll in vivo published by Schwartz [16], the incident intensity $I_0$ at which there is one photon hit ($X = 1$) per photosynthetic unit/ns has been calculated (assuming approx. 300 chlorophyll molecules/photosynthetic unit) and is indicated in Fig. 1B (The intensity at which $X = 1$ in the picosecond pulse case is also given in Fig. 1A for comparison). Since the lifetime of $S_1$ excitons is 0.8 ns at 100 K [17], not more than 30 % of the drop in $\phi$ can be attributed to $S_1 + S_1$ annihilation in the microsecond pulse case when $I_0 \cong 3 \cdot 10^{17}$ photons $\cdot$ cm$^{-2}$ and $X = 1$, since $\phi_{X=1} \cong 0.70\,\phi_{X\to 0}$ in the picosecond pulse case It is evident from Fig. 1B that the extent of quenching is much greater than 30 %, particularly for the Photosystem I fluorescence at 735 nm when $X = 1$. Detailed arguments presented elsewhere [13] indicate that this preferential quenching when microsecond exciting pulses are used are due to triplets ($T_1$) according to the process

$$S_1 + T_1 \xrightarrow{\gamma'} S_0 + T_n \tag{2}$$

where $T_n$ denotes a triplet in a higher excited state [15]. On microsecond time scales there is sufficient time for a buildup of these long-lived quenchers, the lifetimes of which have been shown to be longer than approx. 10 ns [12]. Since the quenching efficiency is considerably greater at 735 nm than at 685 nm, we conclude that a greater density of triplet quenchers is built up within the Photosystem I pigments than within the light-harvesting or Photosystem II antenna light-harvesting pigments on the microsecond timescales. Shuvalov [18] has recently provided evidence which supports our conclusion that long-lived (millisecond) triplets accumulate in Photosystem I

In the single picosecond pulse case, the quenching is completely different than in the microsecond case since there is no difference between the 685 and 735 nm quenching curves. The time scales in these experiments are less than 1 ns and only singlet-singlet annihilation is operative

If we assume that the 685 and 735 nm emission originates from different antennae pigment systems and that singlet-singlet annihilation occurs within both systems, it is difficult to explain why the two quenching curves in Fig. 1A are identical. The reasons for this are the following: (1) the lifetimes of the singlet excitons are different in the two photosystems, the decay at 735 nm being 1.5 ns, while the decay time determined at 685 nm is 0.8 ns at 100 K [17]. A more efficient quenching at 735 nm is thus expected. (2) There is a greater pigment heterogeneity in the light-harvesting pigments (approx. 1 · 1 chlorophyll *a* : *b* ratio) than within the Photosystem I pigments; on this basis Swenberg et al. [19] have proposed that the exciton diffusion coefficients are smaller in Photosystem II than in Photosystem I. If this is the case, then $\gamma$ in (1) and the efficiency of singlet-singlet annihilation should be lower within the light-harvesting and Photosystem II antennae pigments [12, 13] than within the Photosystem I pigments. (3) The distribution of energy and thus the density of singlet excitons may be different within the Photosystem I and Photosystem II light-harvesting antennae pigments. All these factors should give rise to differences in the picosecond pulse quenching curves observed at 685 and at 735 nm. Such differences are not observed. It is highly unlikely that all of the above-mentioned factors should fortuitously combine in such a way as to cancel all these differences, and to produce identical quenching factors at 685 and at 735 nm.

We propose instead that the identical quenching efficiencies at 685 and at 735 nm in the single picosecond pulse case are due to the mutual annihilation of singlet excitons on the level of the light-harvesting antenna chlorophyll-protein complexes. Furthermore, we propose, in accord with previous suggestions [1–3, 20], that the 735-nm emitting pigment forms constitute a relatively small fraction of chlorophyll in vivo and that they derive their energy predominantly by energy transfer from light-harvesting pigments, rather than by direct absorption of photons. Thus the Photosystem I pigments receive a fraction of the excitons which diffuse within the light-harvesting pigment systems. As the annihilations decrease the average density of singlets within the light-harvesting pigment system, fewer excitons eventually arrive at the Photosystem I pigments. In this model, the Photosystem I pigments sample the exciton density within the light-harvesting pigment system. Identical quenching curves are thus predicted for the 685 light-harvesting and the 735-nm Photosystem I emission bands.

In the microsecond pulse case, most of the quenching is attributed to annihilation of singlet excitons by triplets [13]. Since the quenching curves at 685 and 735 nm are different, it appears that the density of these triplets is higher within the Photosystem I pigments than within the light-harvesting pigments (also $\gamma'$ (eqn. 2) in Photosystem I may be higher than in the light-harvesting pigments [13]). If the triplets were present only within the light-harvesting pigments, the two quenching curves would be expected to be the same in the microsecond pulse case. A preferential buildup of triplets in Photosystem I on the timescale of microseconds may be due to their longer lifetimes in this photosystem [18]; it is also possible that triplets which are formed within the light-harvesting pigment complexes are rapidly transferred to the lower-energy Photosystem I pigments. These possibilities are presently being investigated

The overall results are thus consistant with the model of singlet exciton generation within the light-harvesting pigments, their possible quenching by other singlets and subsequent flow to Photosystem I pigments. Triplets, which preferentially build up

in Photosystem I can efficiently quench the singlets which arrive there, thus accounting for the differences between the picosecond and microsecond pulse cases.

This model of energy distribution within the different pigment systems of chloroplasts predicts that the lifetimes of the singlet excitons should remain unchanged in Photosystem I with increasing intensity of the picosecond laser flashes. This is expected if there is no $S_1$–$S_1$ annihilation on the level of the Photosystem I pigments At 685 nm on the other hand, a strong variation of the decay times should be observed as a function of the intensity of the picosecond pulses Accordingly we have measured

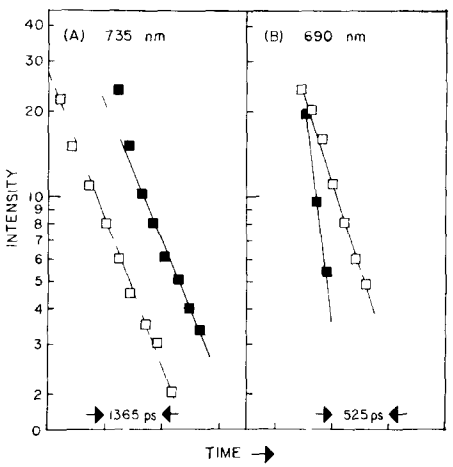


Fig 2 (A) Semilogarithmic plots of the fluorescence decays of spinach chloroplast fluorescence at 77 K. The fluorescence was viewed with interference filters centered at (A) 735 nm and (B) at 690 nm. (A) 735 nm □–□, 3 5 $10^{14}$ photons $cm^{-2}$ per picosecond pulse at 530 nm. ■–■, 2 6 $10^{15}$ photons $cm^{-2}$. The lifetimes estimated from both plots are 1 1 ns. For clarity of presentation the horizontal axes of the two decay curves have been shifted relative to each other (B) 690 nm □–□, 2 5 $10^{14}$ photons $cm^{-2}$, estimated lifetime is 0 38 ns ■–■, 7 4 $10^{14}$ photons $cm^{-2}$, the decay curve is limited by the resolution of the apparatus and the lifetime is thus $\leq$ 0 13 ns.

fluorescence decay curves at 685 and 735 nm as a function of intensity While the expected strong variation in the lifetime of the excitons is observed at 685 nm, little or no variation can be detected when the fluorescence is viewed at 735 nm. Typical decay data taken from actual polaroid photographs are shown in Fig. 2 at several different intensities. Viewing the fluorescence at 735 nm, the lifetime is estimated to be 1.1 ns at both intensities of $3\,5 \cdot 10^{14}$ and $2.6 \cdot 10^{15}$ photons $\cdot$ $cm^{-2}$ per flash At 685 nm on the other hand, the lifetime is 0 38 ns at $2\,5 \cdot 10^{14}$ and less than 0 13 ns (resolution limited) at $7\,4 \cdot 10^{14}$ photons $\cdot$ $cm^{-2}$ per flash. At an intensity of $2\,5 \cdot 10^{14}$ photons $\cdot$ $cm^{-2}$, the relative quantum yield $\phi$ is decreased to 0 57 $\phi_0$, where $\phi_0$ is the maximum yield at the lowest intensities (Fig. 1A). The extrapolated lifetime in this low intensity range is thus 0.38/0 57 $\simeq$ 0 7 ns, which is in reasonable agreement with the results of Hervo et al. [17], who obtained 0.8 ns using a photon counting technique The fact that the intensity measurements (Fig. 1A) were done at 100 K, while the lifetime measurements were done at 77 K reflects only experimental convenience dictated by

the type of cryogenic equipment available in the two laboratories. It is shown elsewhere [21] that the quantum yields at 685 and 735 are identical in the range of 20–200 K. Thus, the basic conclusions are not affected by the slightly different temperatures utilized in obtaining the results depicted in Figs. 1 and 2

The lack of a strong intensity dependence of the lifetime of the 735 nm fluorescence in the intensity range studied thus supports the conclusion that the pigments responsible for this fluorescence derive their energy from other antennae light-harvesting chlorophyll molecules. This implies that these long wavelength pigments themselves have a relatively small optical absorption crossection at 610 nm and are thus present in low concentrations. This latter conclusion is consistent with the computer analysis of the absorption and emission spectra of chlorophyll in vivo [22, 23]. It is commonly accepted that the 735 nm emission at low temperatures originates from Photosystem I While our results are not inconsistent with this assignment, it remains to be proven that the 735 nm emission is indeed derived from pigment complexes which are directly associated with the *P*-700 reaction centers of Photosystem I. In our opinion, the possibility that the 735 nm fluorescence band is due to isolated pigments which are not connected to *P*-700 cannot be excluded at this time.

Finally, it is well known that upon steady state illumination the fluorescence intensity increases on the time scale of seconds: this time-dependent fluorescence enhancement (induction) is lower at 735 than at 685 nm and is attributed to the closing of the reaction centers in Photosystem II and the spillover of excitons from Photosystem II to Photosystem I [5, 24]. Spillover involves the energy flow pathways Photosystem II → light-harvesting Photosystem I. If the Photosystem II → light-harvesting complex pathway is less efficient than the light-harvesting complex → Photosystem I pathway, then an increase in the exciton density within Photosystem II leads to a smaller increase of excitons within Photosystem I, thus accounting for the wavelength dependence of the induction effect [24]. In our picosecond pulse quenching experiments only the pathway light-harvesting complex → Photosystem I is involved; thus, in our case the same changes in the fluorescence yield are expected at 735 and 685 nm, as long as the singlet-singlet annihilations occur on the level of the light-harvesting complex pigments with subsequent flow of excitons to Photosystem I. Thus, there is no inconsistency between the wavelength dependence of the fluorescence induction effect and the model which is proposed here to account for the picosecond pulse fluorescence quenching experiments. These effects are discussed in greater detail elsewhere [21].

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