Biochimica et Biophysica Acta 808 (1985) 66–76
Elsevier

BBA 41762

Domain sizes in chloroplasts and chlorophyll-protein complexes probed by fluorescence yield quenching induced by singlet-triplet exciton annihilation

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(Received October 8th, 1984)
(Revised manuscript received February 13th, 1985)

Key words: Chlorophyll-protein complex; Fluorescence quenching; Exciton annihilation; (Spinach chloroplast)

Utilizing about 1 μs duration laser pulse excitation at 650 nm, it is demonstrated that the phenomenon of singlet–triplet exciton annihilation can provide information on the domain size of photosynthetic systems (number of chlorophyll molecules connected to each other by energy transfer). The domain sizes are estimated from the shapes of the fluorescence yield vs. excitation-energy curves, from the number of photon hits per molecule per pulse, and from the triplet quantum yield utilizing the master equation theory developed by Paillotin, Geacintov and Breton (Paillotin, G. et al. (1983) Biophys J. 44, 65–77). The photosynthetic systems investigated in this manner include light-harvesting chlorophyll-protein complexes (LHCP) isolated by sodium dodecyl sulfate solubilization of spinach chloroplasts followed by polyacrylamide gel electrophoresis, aggregates of light-harvesting complexes (LHC), and Photosystem I particles (both prepared by Triton X solubilization and sucrose gradient centrifugation), as well as chloroplasts and free chlorophyll *a* in pyridine solution or polyacrylamide gels. The LHCP particles are characterized by approx. five connected chlorophyll molecules per domain and a triplet quantum yield of approx. 0.2. The LHC complexes and chloroplasts are examples of large domains (more than 240 molecules) with triplet quantum yields of approx. 0.08 and 0.10–0.15, respectively. The Photosystem I particles similarly constitute large domains, but the fluorescence quenching is relatively inefficient, because of the apparently low triplet quantum yield of approx. 0.01.

Introduction

A photosynthetic domain is defined as a collection of n chlorophyll molecules, over which excitons can range in a photosynthetic system [1,2]. In the case of small chlorophyll-protein complexes extracted from chloroplasts by treatment with detergents [3], the relatively small number of chlorophyll molecules form a domain with physical boundaries which confine the motion of the excitons. However, in the case of intact thylakoid membranes in chloroplasts there appear to be no such physical boundaries [4], and the domain size can be loosely defined as the number of pigment molecules over which the excitons can range during their lifetimes.

Bimolecular exciton annihilation processes result from the interactions of two excitons and can,

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Abbreviations: LHCP, light-harvesting chlorophyll *a*,*b*-protein; LHC, light-harvesting chlorophyll-protein; PS I, Photosystem I; OMA, optical multichannel analyzer.

in principle, provide information about the size of the domains. Annihilation phenomena result in a decreased fluorescence yield, and, depending on the system, a decrease in the fluorescence decay time may or may not also be observable [1]. The size of the domains plays a role in annihilation effects, because at least two excitations must be present in a domain if annihilation is to occur. At a given level of excitation intensity, the probability of creating two excitations in a small domain is much lower than the probability of creating two excitons in a larger domain [5]. Thus, the onset of fluorescence quenching with increasing excitation energy occurs at lower energies for large domains more than for small domains, all other conditions being equal.

Two types of exciton–exciton annihilation phenomena have been identified: singlet–singlet and singlet–triplet annihilation. The former process is favored in picosecond laser pulse excitation experiments, while singlet–triplet annihilation is favored when the rate of creation of singlet excitons is much lower than their decay rate; thus singlet–triplet annihilation is favored in microsecond duration laser pulse experiments [5].

Paillotin et al. [1] have formulated a master equation theory which relates the excitation intensity dependence of the fluorescence yield in photosynthetic systems to domain sizes. This theory was applied successfully by Van Grondelle et al. [6] to determine the size and structure of antenna complexes of photosynthetic bacteria. These workers have more recently also formulated a detailed theory of singlet–singlet annihilation and have included random walk and the trapping of excitons by open or closed reaction centers in their formulation [7,8].

Singlet–triplet exciton annihilation has been investigated in photosynthetic bacteria by Monger and Parson [9] and by Breton et al. in chloroplasts [10] and in isolated light-harvesting chlorophyll-protein (LHCP) complexes [11]. In Ref. 10, the dependence of the fluorescence yield on laser pulse energy was interpreted in terms of a set of coupled differential equations, which were solved numerically. Clearly, such an approach is applicable only in the case of large domains in which the exciton density can be approximated by a continuous variable function. In the case of small domains in which the number of excitons per domain is finite, this approach is not valid and Paillotin et al. have formulated a detailed master equation theory to describe singlet–triplet annihilation phenomena in domains of various sizes [2]. In this work we have explored the applications of this theory to studies of singlet–triplet exciton annihilation in photosynthetic systems of different domain sizes consisting of spinach chloroplasts and submembrane fractions derived from chloroplasts. It is shown that fluorescence yield versus excitation-energy curves can provide semiquantitative estimates of domain sizes, provided the quantum yield of triplet-excited states can be estimated from fluorescence decay times, or from other data.

Theoretical aspects

Singlet–singlet annihilation can be neglected when microsecond laser excitation pulses are utilized because the probability of two singlet excitons encountering one another is negligible [11]. Singlet excitons have a finite probability to decay to chlorophyll triplet excitons; the latter transfer their energy to carotenoid molecules to form carotenoid triplet-excited states (rate constant, approx. $1 \cdot 10^8\ s^{-1}$ and quantum yield approx. 10–15% in chloroplasts [12]). As time increases after the onset of the laser excitation pulse, the density of carotenoid triplets increases [10], since their lifetime of several microseconds is longer than the duration of the laser pulse (approx. 0.7 μs half width at half maximum in our experiments). In the theory of Paillotin et al. [2], a domain is assumed to contain m carotenoid molecules. Each of these can be transformed into a triplet-excited state which, subsequently, can quench singlet excitons, and thus lower the fluorescence yield. The number of carotenoid triplet quenchers per domain is denoted by i, where $0 \leqslant i \leqslant m$.

The other parameters in the theory are R, X and Y which are defined as follows:

$$R = \frac{K_0}{K_m} \tag{1}$$

where K_0 and K_m are total rates of deactivation of singlet excitons in domains which contain no carotenoid triplet quenchers ($i = 0$), and the maxi-

mum number of triplets ($i = m$) respectively. Furthermore, if I is the fluence incident on the sample, and σ_n is the absorption cross section per domain, Y is defined as the integrated number of photons absorbed per pulse per domain:

$$Y = \int_0^\infty \sigma_n I \, dt \tag{2}$$

The parameter X is the number of absorbed photons per carotenoid molecule which are converted to triplet excited states; it is a function of the quantum yield of triplet formation χ_0 and is defined by:

$$X = \chi_0 Y/m \tag{3}$$

The experimentally measured fluorescence signal is F (integrated over the entire laser pulse), and the fluorescence yield is defined as:

$$\Phi(Y) = F/Y \tag{4}$$

In the limit of low Y ($i = 0$; no fluorescence quenching), the fluorescence yield is Φ_0, while in the limit $Y \to \infty$ ($i = m$: maximum quenching), the yield is denoted by Φ_m. We note that the parameter R in eqn. 1 can also be defined in terms of the yields such that $R = \Phi_m/\Phi_0$.

In the master equation theory [2] it is useful to calculate a normalized fluorescence yield Φ' rather than the experimentally observable yield Φ. These quantities are related as follows:

$$\Phi'(X) = \frac{\Phi(X) - \Phi_m}{\Phi_0 - \Phi_m}$$

$$= \frac{\Phi(X)/\Phi_0 - R}{1 - R} \tag{5}$$

Experimentally it is convenient to measure the relative fluorescence yield $\Phi(X)/\Phi_0$, and thus the limits of the normalized yield $\Phi(X)$ are:

$$0 < \Phi'(X) < 1.0 \tag{6}$$

and those of $\Phi(X)/\Phi_0$ are:

$$R < \Phi(X)/\Phi_0 < 1.0 \tag{7}$$

and we can rewrite Eqn. 5 as:

$$\Phi/\Phi_0 = (1 - R)\Phi'(X) + R \tag{8}$$

which relates the experimentally observed yield $\Phi(X)/\Phi_0$ to the theoretically calculated yield $\Phi'(X)$. In these equations $R < 1.0$, and R depends on the fluorescence quenching efficiency of carotenoid or other triplets. At high excitation intensities, X, the relative yield $\Phi(X)/\Phi_0$ is expected to approach the value of R. Of course, if $R = 0$, then $\Phi(X)/\Phi_0$ approaches zero as $X \to \infty$.

The theoretical yield $\Phi'(X)$ can be calculated as a function of the parameter X according to the following equation [2]:

$$\Phi'(X, m, R) = \frac{1}{X}\left[m - K \sum_{j=0}^{j=m-1} \frac{m!}{(m-j)!j!} \frac{(1-R)^{m-j-1}}{m^{m-1}} \right.$$

$$\times (m-j)^{m-j-1} (j + (m-j)R)^j$$

$$\left. \times \exp\left\{ \frac{-(m-j)X}{m-j+j/R} \right\} \right] \tag{9}$$

This equation can be conveniently evaluated by computer, utilizing m and R as parameters. Some typical $\Phi'(X)$ curves utilizing $R = 0.03$ and calculated for some different values of m are shown in Fig. 1.

There are two important limits of Eqn. 9, one for very large domains ($m \to \infty$), and the other for very small domains ($m = 1$). In the large domain case [2] and for small values of R (efficient quenching by carotenoid triplets):

$$\Phi'(X) = \frac{\sqrt{1 + 2\frac{X}{R}} - 1}{\frac{X}{R}} \tag{10}$$

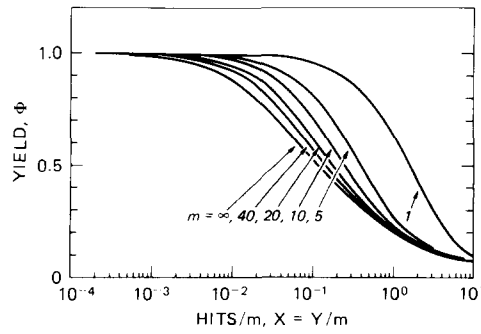

Fig. 1. Fluorescence yield calculated according to Eqn. 9 for different values of the parameter m (number of potential triplet quenchers/domain) for a fixed value of the parameter $R = 0.03$.

A plot of this equation with $R = 0.03$ is identical in shape and position along the horizontal axis to the $m = \infty$ curve shown in Fig. 1.

In the limit of small domains ($m = 1$), and for all values of R, Eqn. 9 reduces to:

$$\Phi'(X) = \frac{1}{X} \quad (1 - e^{-X}) \tag{11}$$

This equation is also obtained from a simple consideration of a Poisson distribution of photon hits per domain, in which all hits, except for the first one, contribute nothing to the fluorescence [13].

Experimental methods

Sample preparation

Spinach chloroplasts were prepared according to the procedures outlined in Ref. 14, and were suspended in 0.4 M sucrose-Tris buffer solution (50 mM, pH 8.0). These preparations exhibited the usual absorption maximum at 677–678 nm and a fluorescence emission maximum at 685 nm.

The light-harvesting chlorophyll-*a*/*b*-protein (LHCP) complex described by Thornber [15] was isolated from chloroplasts by the method described by Van Metter [16]: this procedure involved the solubilization of chloroplasts in sodium dodecyl sulfate-Tris buffer, 5 mM $MgCl_2$ solution, and subsequent electrophoresis on polyacrylamide gels at room temperature. Three bands were obtained, and a slice of the central band was cut out from the gel and squeezed between two microscope slides until the desired optical density at the laser excitation wavelength selected was obtained. These preparations exhibited absorption maxima in the Soret region at 672 and at 652 nm, and the 672 nm/652 nm absorbance ratios varied in the range 1.15–1.20; this ratio indicates that the chlorophyll *a*/chlorophyll *b* ratio is close to unity. It has been previously suggested that each protein contains approx. three chlorophyll *a* and three chlorophyll *b* molecules [15,16].

Experiments were also performed utilizing the electrophoretically most mobile band due to free pigments; the corresponding gel slice was also squeezed between two microscope slides until the desired optical density at the excitation wavelength was obtained. In other experiments, purified chlorophyll *a* (Sigma Chemicals, St. Louis, MO) was dissolved in pyridine and the solutions were placed in 1 mm pathlength cuvettes. Thus, the fluorescence properties of free chlorophyll molecules either in the gels or in solution exposed to laser excitation were compared to those of the chlorophyll protein complexes.

Aggregates of light-harvesting chlorophyll-protein (LHC) complexes were prepared from spinach chloroplasts by the methods of Burke et al. [17]. Absorption maxima at 675 and 652 nm were observed, and the ratio of 675/652 absorbance was approx. 1.45. Appropriate dilutions were performed with Tris buffer and these samples were also contained in 1 mm pathlength cuvettes during the experiments.

Photosystem I particles (PS I) were prepared by Triton solubilization and sucrose gradient centrifugation according to methods previously described by Mullet et al. [18]. An absorption maximum at 676–677 nm was observed, and shoulders at about 630 and 590 nm (about 3.8- and 6.5-times lower in amplitude, respectively, than the 676–677 nm peak) were also evident. These preparations are believed to represent the Photosystem I antenna complexes and are characterized by a ratio of chlorophyll/P-700 of 110 ± 10, and consist of 10–13 nm diameter protein complexes which are believed to be aggregated [18].

Instrumentation

A schematic outline of an apparatus similar to the one used in this work is shown in a previous paper [10]. A Chromatix CMX-4 (Chromatix Corp., Sunnyvale, CA) xenon flash lamp-pumped dye laser (Rhodamine 640) operating at 650 nm was utilized to excite the fluorescence of the samples. Utilizing a boxcar averager (Princeton Applied Research, Princeton, NJ, Model 610 main frame with Model 162 plug-in units, 3 ns/point time resolution), the time-dependent intensity profile of the excitation laser pulse was determined; a Hamamatsu R928 photomultiplier tube (Hamamatsu Corp., Middlesex, NJ) was used as a detector in these experiments. In this manner the full width at half maximum of the laser pulse was found to be 700 ns [19].

In this work it was necessary to determine the number of photons per unit area per pulse as

accurately as possible. Therefore the spatial beam profile was measured utilizing a photodiode mounted behind a 0.005 cm diameter pin hole; this assembly was in turn mounted on a micrometer-operated $X - Y$ stage, which was oriented with its plane vertical to the direction of the laser beam. The pin hole-photodiode assembly was displaced in both directions and the average intensities of the laser pulses were recorded at each position; in this manner, a two-dimensional representation of the beam profile was obtained (Fig. 2A); the diameter of the beam was estimated as shown in Fig. 2B, from which a beam area of $2 \cdot 10^{-3}$ cm^2 was obtained. Because the energy of the pulse falls rather sharply near the boundaries, the inhomogeneities in the beam profiles were neglected. A correction for this effect [20] would be smaller than the other systematic errors inherent in these experiments.

The intensity of the laser beam was varied by placing individual (or stacks of) calibrated neutral density filter(s) between the laser and the sample. The transmittance of each stack of filters was independently determined by placing a photodiode or energy meter at the location of the sample.

The microscope slides or 1 mm cuvettes were oriented at 45° to the incident laser beam and the fluorescence was focussed, by means of two lenses, onto the entrance slit of a flat-field spectrograph (Jobin-Yvon Model UV200, $f = 4.5$, Instruments SA, Inc., Metuchen, NJ). The signal was detected

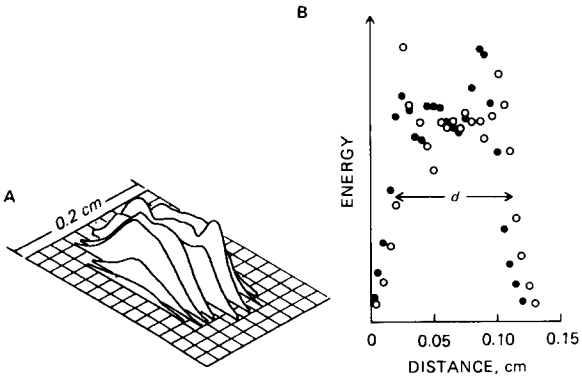

Fig. 2. (A) Example of a laser beam profile determined with a 0.005 cm diameter aperture. (B) Energy variations along two perpendicular directions (● and ○) through the center of the beam in part (A). The effective area of the beam was calculated utilizing an estimated value of the diameter $d = 0.09$ cm.

by an optical multichannel analyzer (OMA) system consisting of a 1205D Vidicon detector and a 1205A console (Princeton Applied Research, Princeton, NJ). The output of this OMA system was fed into a display oscilloscope which allowed for the viewing of the full fluorescence spectrum of the sample after each laser shot, or the viewing of the averaged fluorescence spectra after a large number of laser shots. The relative intensities of the fluorescence signals as a function of the laser beam intensities were evaluated for an appropriately averaged signal displayed in digital form by the OMA console. In order to avoid overload of the vidicon detection system at the higher laser intensities, stacks of calibrated neutral density filters were inserted as necessary between the sample and the entrance slit of the spectrograph.

The energy of the laser beam was monitored in two different ways: (1) utilizing a 45° beam splitter and a calibrated photodiode, and (2) a portion of the laser beam scattered from the sample was also allowed to reach the spectrograph; thus, in each experiment the intensity of the attenuated laser line at 650 nm relative to the intensity of the fluorescence signal in the 675–685 nm region of the spectrum could be determined.

The laser beam intensity dependence of the transmittance of the samples was also measured. This was achieved by placing a photodiode behind the samples along the direction of the laser beam. By determining the intensity of the transmitted light (integrated over the duration of the laser pulse) relative to the intensity of the incident light, it was possible to detect any bleaching effects which may have occurred at high laser pulse energies.

Results and Discussion

The fluorescence yield Φ in this work is defined as the ratio of the fluorescence signal integrated over the entire laser pulse width divided by the total energy of the pulse incident on the sample. The data are represented in terms of fluorescence quenching curves which are plots of Φ as a function of the fluence I. Typical data for free pigments, LHCP and LHC are shown in Fig. 3A, and for chloroplasts and PS I particles in Fig. 3B.

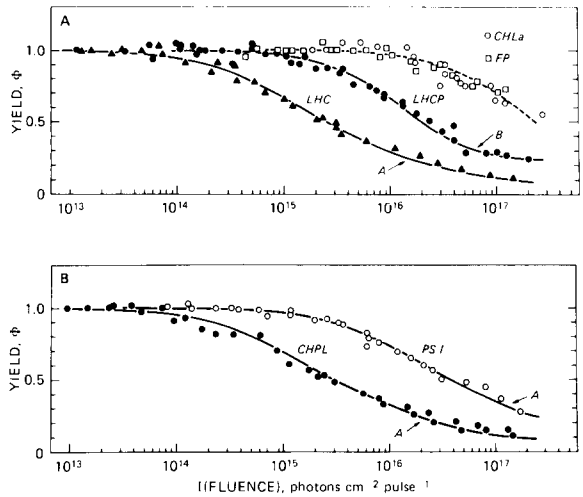

Fig. 3. (A) Fluorescence yield as a function of the incident fluence (650 nm excitation). ○, $6 \cdot 10^{-5}$ M chlorophyll *a* in pyridine (air saturated). Absorbance (650 nm) = 0.05; □, free pigment band on gel slice; ●, LHCP, chlorophyll-protein complexes on gel slice. The points represent data obtained with three different samples (absorbance values, 0.28, 0.35 and 0.46 at 650 nm). ▲, LHC, light harvesting chlorophyll protein aggregate in suspension (1 mm cuvette). Data obtained with two different samples (absorbance, 0.30 and 0.40 at 650 nm). Curve A: plot of Eqn. 10, adjusted along the horizontal axis to provide the best fit to the experimental data. Curve B: plot of Eqn. 13 and adjusted along the horizontal axis to fit the experimental data. (B) Fluorescence yield as a function of incident fluence for chloroplasts (CHPL) and PS I preparation (650 nm excitation). The points represent data obtained with two separate samples of each type of preparation (absorbance, 0.13, 0.33 for CHPL and 0.22 and 0.21 for PS I at 650 nm, respectively). Both curves (A) are plots of Eqn. 10 adjusted along the horizontal axis to provide the best fit to the experimental data.

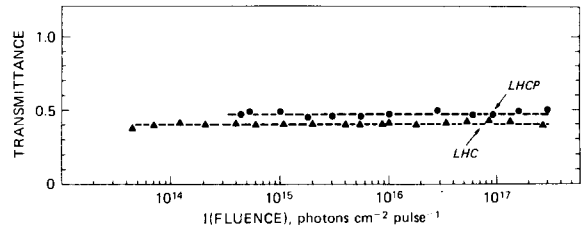

Fig. 4. Transmittance (integrated over the entire duration of the laser pulse at the excitation wavelength of 650 nm) of LHCP and LHC samples as a function of the fluence.

While the fluorescence yield exhibits a prominent decrease as a function of increasing fluence, there was no observable change in the transmittance of the chlorophyll-protein samples under the same conditions of excitation; this is illustrated for the LHC and LHCP samples in Fig. 4. These results show that the observed drop in Φ is not due to a depopulation of ground state pigment molecules subjected to intense irradiation, and can be attributed to exciton annihilation [21,22]. In the case of the free pigments and dilute chlorophyll *a* solutions, however, the drop in Φ with increasing fluence (Fig. 3A) can be attributed to ground-state depletion. Changes in the transmittance of such dilute chlorophyll *a* (in pyridine) solutions have been observed [19] and occur at the same levels of excitation at which decreases in Φ are also evident. Thus, the photophysical basis of the fluorescence quenching curves observed with the free pigments is quite different from the exciton annihilation phenomena which give rise to the decreases in Φ with increasing Y in the case of the LHCP, LHC, PS I and chloroplast samples.

It is of interest to compare the relative excitation levels at which the fluorescence yields Φ begin to decline for the different preparations. This is best achieved by evaluating the quantity σI, which is equal to the number of photons absorbed per molecule per pulse, for each type of sample where $\sigma = 3.81 \cdot 10^{-21} \cdot \epsilon$ cm^2 is the molecular absorption cross section at the excitation wavelength and ϵ is the molar absorption coefficient. Values of ϵ are available in the literature for chlorophyll *a* [23], LHCP [24], PS I [25] and chloroplasts [26], and we have utilized these values and absorption spectra to estimate σ at 650 nm for the different preparations. The following values of the cross-sections at 650 nm were utlized: $0.6 \cdot 10^{-16}$ (chloroplasts), $1.1 \cdot 10^{-16}$ (LHCP and LHC), $0.8 \cdot 10^{-16}$ (PS I) and $0.95 \cdot 10^{-16}$ cm^2 (chlorophyll *a* in pyridine). Schematic representations of the fluorescence quenching curves as function of σI are compared in Fig. 5. We arbitrarily denote the onset of quenching by the σI values at which the yields Φ are reduced to 0.9; this onset occurs when there are approx. 0.3 hits per molecule in the case of LHCP, only approx. 0.01–0.02 hits per molecule in the case of LHC and chloroplasts, and approx. 0.2 hits per molecule in the case of the PS I particles.

This comparison constitutes a qualitative illustration of the domain size effect [5,11]. The LHCP

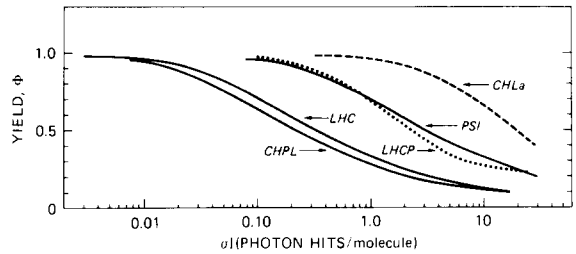

Fig. 5. Plots of fluorescence quenching curves for the different preparations in Fig. 3 and 4 as a function of absorbed photon hits per molecule. (σI).

complexes in this preparation contain about six chlorophyll molecules, and about two hits per chlorophyll-protein complex per pulse are necessary to observe a decrease in the yield. In the case of the LHC preparations and chloroplasts, the chlorophyll-protein complexes are aggregated; this gives rise to exciton transfer between complexes and thus to a larger domain size. Thus, comparing the σI values for the LHCP and LHC system, it appears that the domain size (number of molecules per domain) is at least $0.3/0.02 \approx 15$ times larger for LHC (and chloroplasts) than for the complexes. Such comparisons, however, are only approximate, since the shapes of the fluorescence quenching curves depend on domain size (Fig. 1), and the yield of triplets may be quite different in the different systems. Thus, the position of the Φ curves with respect to the fluence depends not only on Y, the number of absorbed photon hits per domain, but also on the triplet yield per quencher, χ_0/m. A more detailed analysis of these effects is therefore necessary.

Dilute chlorophyll a solutions and free pigments

In these systems the chlorophyll molecules are far apart from one another, and thus no energy transfer between different molecules, or bimolecular excited state annihilation, can occur. Therefore the behavior of dilute chlorophyll solutions, specifically variations of Φ as a function of the fluence, can be modeled by a set of coupled first-order differential equations describing the time dependence of the concentrations of ground state, first singlet-excited state and triplet-excited molecules [19]. Brandimarte [19] has analyzed such systems in detail, utilizing the same type of laser pulse excitation as in this work, by solving this set of differential equations numerically and fitting the fluorescence quenching curves to experimental Φ vs. I and transmittance vs. I data ($5 \cdot 10^{-5}$ M chlorophyll a in air-saturated pyridine). Excellent fits were obtained, and it was shown that the decrease in Φ with increasing fluence can be attributed mostly to the promotion of the chlorophyll a molecules to the triplet excited state, thus leading to a significant depopulation of ground state and singlet excited states. This phenomenon can occur with the type of laser excitation used in this and Brandimarte's work, because the triplet lifetime of 430 ns observed in air-saturated pyridine solutions (Mathis, P., private communication) is comparable to the laser pulse width of approx. 700 ns. We shall not further analyze the dilute chlorophyll a case, since details have been described elsewhere [19]; we note only that the behavior of the fluorescence yield vs. I curves for the free pigments dispersed in the gel slices is the same, within experimental error, as the fluorescence yield curves for the dilute chlorophyll a solutions.

Light-harvesting chlorophyll-protein complexes

In the case of the LHCP complexes which are small in size, Eqn. 11 should be the most suitable one to describe the fluorescence yield as a function of the excitation energy. However, before attempting to fit this equation to the experimental results, it is necessary to consider the photophysical processes which occur in these chlorophyll-protein complexes in more detail.

The LHCP particles are believed to contain at least one carotenoid molecule [27]. We assume that only one triplet excited state can be created per protein complex and thus $m = 1$. The lack of any changes in the transmittance at the excitation wavelength (Fig. 4) suggests that there is no significant depopulation of ground state chlorophyll molecules during the pulse; as in chlororoplasts [12], chlorophyll singlets are rapidly converted to chlorophyll triplets, which in turn are rapidly quenched by energy transfer to carotenoid molecules. Consistent with this picture, Shepanski et al. [28] have found that formation of carotenoid triplets in LHCP particles occurs within less than 5 ns after excitation with a laser pulse (4 ± 1 ns

width) within the chlorophyll absorption band; furthermore, they determined that the lifetime of these carotenoid triplets is 8.7 ± 0.5 μs. The lifetime of the quencher is thus about 10 times longer than the laser pulse width, justifying one of the critical assumptions made in the derivation of Eqn. 8.

The connection between the experimentally determined fluorescence quenching curves and the theoretically derived equations is made via Eqn. 8, utilizing either one of the Eqns. 9–11. In the case of the LHCP complexes which constitute a small domain, Eqn. 11 is inserted into Eqn. 8 and the best fit to the data is obtained with $R = 0.2$ (Fig. 3A) according to the equation:

$$\Phi(X)/\Phi(0) = 0.8\ \Phi'(X) + 0.2 \tag{13}$$

This equation implies that the fluorescence yield of LHCP complexes is 20% when there is one carotenoid triplet per complex. However, carotenoid triplets are known to be very efficient quenchers of the chlorophyll fluorescence [10,28,29–31], and it is thus unlikely that there would be such a large residual fluorescence (approx. 20%) in the relatively small LHCP particles which contain only about six chlorophyll molecules per carotenoid triplet. Another explanation for the residual 20% fluorescence may be the presence of one unconnected or poorly connected chlorophyll molecule. The presence of such uncoupled or weakly coupled molecules may be due to the detergent treatment and solubilization of the chlorophyll-protein complexes during their isolation. Such treatment may perturb the conformations of at least some of the pigment molecules, thus giving rise to a background fluorescence of approx. 20% which is not subject to quenching by exciton–exciton annihilation. Searle et al. [32] have recently measured the fluorescence decay profile of chlorophyll-*a*/*b* protein complexes isolated from barley by solubilization with sodium dodecyl sulfate and gel electrophoresis (similar to our isolation procedure). They find that the fluorescence decay profile of their preparations is characterized by two components, one with a lifetime of 2.55 ns and an amplitude of 80%, and the other with a lifetime of 4.55 ns and an amplitude of 20%. This latter component exhibits decay kinetics similar to those of free chlorophyll molecules, and is thus likely to be due to one unconnected chlorophyll molecule/protein complex. The presence of one such unconnected molecule in our LHCP complexes could thus easily account for the 20% background fluorescence.

The validity of applying Eqns. 8 and 11 to the interpretation of the LHCP fluorescence quenching curves can be further tested by determining if the values of χ_0, the carotenoid triplet quantum yield, are reasonable. When $X = 1$, the value of $\Phi'(X) = 0.63$, and the value of $\Phi(X)/\Phi(0)$ is 0.71 (from Eqn. 13). Such a reduction in the yield occurs when the fluence $I = 8 \cdot 10^{15}$ photons $\cdot$ cm^{-2}. We can thus write;

$$X = 1.0 = \chi_0 \frac{n}{m} \sigma I \tag{14}$$

where we have substituted $n\sigma$ for σ_n, the cross-section per domain, and where $n \approx 5$ is the number of (connected) chlorophyll molecules per domain. Utilizing the value of σ given earlier in this paper and with $m = 1$, we obtain $\chi_0 \approx 0.23$.

The carotenoid triplet yield can be estimated from the fluorescence decay time τ_f and the intersystem crossing constant $k_{is} = 1 \cdot 10^8$ s^{-1} for chlorophyll *a* molecules [33]. This approach is feasible because Bowers and Porter [33] found that the sum of fluorescence and triplet yields is nearly unity in polar solvents, in which chlorophyll molecules do not form oligomeric aggregates [34]. Assuming further that the conversion of chlorophyll triplets to carotenoid triplets is (almost) 100% efficient [12], the relationship between τ_f, k_{is} and χ_0 is:

$$\chi_0 = k_{is}\ \tau_f \tag{15}$$

The value of τ_f for LHCP, or similar complexes has been previously determined by several groups. Lotshaw et al. [35] obtain biphasic decays of 1.2 and 3.3 ns with nearly equal amplitudes; Nordlund and Knox [36] report a value of 3.1 ± 0.3 ns utilizing picosecond laser excitation and a streak-camera technique. Utilizing phase methods, Moya and Tapie [37] report heterogeneous fluorescence in monomeric light-harvesting protein complexes with $\tau_f \approx 2.5$–3.0 ns. Il'ina et al. [38] report a value of approx. 4.1 ns for a monomeric form of a light-harvesting chlorophyll-protein complex iso-

lated by sodium dodecyl sulfate-gel electrophoresis. Utilizing average values of τ_f in the range of 2.5–4.0 ns and utilizing Eqn. 15, values of χ_0 in the range of 0.25–0.40 can be estimated. Therefore, our value of $\chi_0 \approx 0.23$ is consistent with this range of numbers. Shepanski et al. [28] have found experimentally, utilizing a 4 ns wide nitrogen laser excitation pulse, that the triplet quantum yield is only 5%. The reason for this discrepancy is not known, but may be due to differences in the excitation pulse widths.

We conclude that the limiting form of Eqn. 9, as expressed by Eqn. 11, gives an adequate representation of the fluorescence quenching curves observed with LHCP complexes, provided that $\chi_0 \approx 0.2$ and that about 20% of the fluorescence can be attributed to unconnected or 'dead' chlorophyll fluorescence. Van Metter [16] did not observe any contributions of such unconnected molecules in his circular dichroism and fluorescence polarization studies of LHCP complexes. However, fluorescence yields under conditions of exciton annihilation and fluorescence decay profiles [32] are more sensitive to the presence of such minor fluorescence-emitting, unconnected chlorophyll components.

Light-harvesting complexes

The characterics of these preparations have been described by Mullet and Arntzen [39]; sheet-like aggregates of highly organized particulate subunits are observed, which are believed to be individual light-harvesting pigment-protein complexes, with diameters of 6–10 nm. Under the conditions of our experiments (no added $MgCl_2$) the LHC were shown to be interconnected in lamellar sheets, but not stacked on top of one another [39].

As expected for such large aggregates of chlorophyll-protein complexes, the onset of fluorescence quenching occurs at energies which are about 15-times lower than those observed for the individual LHCP complexes. This observation is consistent with a delocalization of the excitons over a domain which is at least 50–60 molecules in size. A more accurate lower limit of the domain size can be obtained by comparing the fits of the different theoretical curves shown in Fig. 1 to the experimental data. The best fits are obtained in the $m \to \infty$ limit and for $R \approx 0$; this is shown by the solid line, which is a plot of Eqn. 10 (or of Eqn. 8 with $R = 0$), superimposed on the experimental LHC data in Fig. 3A. In practice it is difficult to differentiate between the best fits to the experimental data of any of the theoretical curves for which $m > 40$. Thus, since we assume that there are m possible carotenoid tripletes per domain, and the number n/m of chlorophyll molecules per carotenoid molecule is approx. six, the minimum domain size for LHC aggregates is $n \approx 240$.

According to Eqn. 10, $\Phi(X) = 0.732$ when $X/R = 1$. The exact value of R cannot be determined from the experimental data, but it is evident that R must be less than 0.1 (Fig. 3A). Also from Fig. 3A, $X/R = 1$ when $I \approx 7 \cdot 10^{14}$ photons $\cdot$ cm^{-2} per pulse. Using $\sigma = 1.1 \cdot 10^{-16}$ cm^2 (assumed to be similar to the LHCP values at 650 nm), and $n/m = 6$, we obtain;

$$\chi_0 = 2.2\ R \tag{16}$$

since $R < 0.1$, $\chi_0 < 0.22$. According to Nordlund and Knox [36], τ_f in LHC is 1.2 ± 0.5 ns, and Moya et al. [40] give a value of approx. 0.8 ns. Using this latter value of τ_f and Eqn. 15, we estimate that $\chi_0 = 0.08$. Thus the value of R estimated from Eqn. 16 is approx. 0.04, which is consistent with the experimental data.

Chloroplasts

The behavior of chloroplasts is similar to the behavior of the LHC preparations. Eqn, 10 for large domains (and $R \approx 0$) also provides an excellent fit to the experimental data (Fig. 3B). Thus the domain size in chloroplasts is also more than 240 molecules which is consistent with estimates from singlet–singlet exciton annihilation quenching techniques [4,20]. For $X/R = 1.0$, the experimental value of I is $8 \cdot 10^{14}$ photons $\cdot$ cm^{-2}. Chemical analysis shows that there are about five chlorophyll $(a + b)$ molecules per carotenoid molecules [41]. Thus, the value of $n/m \approx 5$–6 is probably as appropriate for chloroplasts as it is for the LHCP and LHC preparations. Utilizing these values and $\sigma = 6.9 \cdot 10^{-17}$ cm^2, we obtained $3.0\ R \leqslant \chi_0 \leqslant 3.6\ R$. The value of χ_0 in chloroplasts depends on the state of the Photosystem II reaction centers and varies from 0.05 to 0.15 [12], the latter value corresponding to closed reaction

centers. In these experiments, the samples were excited with laser pulses at a rate of 10 Hz, and no particular precautions were taken to keep the samples in the dark. Thus the triplet yields are estimated to be in the range of $\chi_0 = 0.10–0.15$ under these conditions. The corresponding values of R are about 0.025–0.050, which again is consistent with the experimental observations of $R < 0.1$.

PS I particles

The position of the fluorescence quenching curve along the horizontal axis is similar for the PS I and LHCP preparations (Figs. 3 and 5); about the same number of photon hits per molecule is required to bring about a similar reduction in Φ. However, the shapes of the two curves are different, the decline in Φ with increasing fluence being more gradual in the case of the PS I particles; Eqn. 10 (or Eqn. 8 with $R \approx 0$) provides a good fit to the experimental data.

The nature of the long-lived fluorescence quenchers in PS I particles at room temperature has not been well characterized. The number of carotenoid molecules per chlorophyll molecule is above $n/m > 20$ in PS I [41]. Thus, the possibility that chlorophyll triplets act as long-lived quenchers in PS I particles cannot be entirely excluded; in that case we note that the ratio n/m would be equal to unity. Since the nature of the quenchers is not known under our experimental conditions, the ratio n/m must be treated as an unknown quantity with $n/m < 20$.

The fluorescence decay time in PS I preparations is known to be quite short at room temperature, about 100 ps or less [42,43]. According to Eqn. 15, the triplet quantum yield is thus expected to be approx. 0.01 or less. This lower yield of triplets is expected to result in a shift of the PS I fluorescence quenching curves to higher excitation energies by a factor of 10–15 with respect to chloroplasts; such a shift is actually observed experimentally (Fig. 3B).

From Fig. 3B, it is found that $X/R = 1$ when the fluence $I = 1 \cdot 10^{16}$ photons $\cdot$ cm^{-2}; noting that $R < 0.3$ (Fig. 3B) we obtain:

$$n/m = \frac{1.2R}{\chi_0} < \frac{0.36}{\chi_0}$$

with $\chi_0 = 0.01$, $n/m < 36$. This value of chlorophyll molecules per potential triplet quencher is within the limits discussed above. Thus, the theory provides an adequate explanation of the data indicating, from the shape of the experimentally observed fluorescence quenching curve and Fig. 1, that $m > 40$. This in turn implies that the PS I particles constitute large domains of aggregated protein complexes [18] with $m > 40$.

Conclusions

Using laser pulses of either picosecond or microsecond duration, singlet–singlet [1,6] or singlet–triplet annihilation can be utilized to estimate the size of photosynthetic systems. In the picosecond method it is necessary to determine accurately the number of excitations per molecule; the shape of the fluorescence yield vs. fluorescence curves (which depend on the ratio of monomolecular to bimolecular decay rates) and the positions of this curve on the fluence axis, provide information on domain size. In the microsecond method, it is necessary to know the triplet quantum yield, as well as the number of excitations per molecule. Again the shapes and positions of the fluorescence yield vs. fluence curves determine the size of the domains. In both cases, only lower limits of domain sizes can be estimated if the size of the photosynthetic systems is greater than approx. 200–250 pigment molecules. In the case of chloroplasts, both methods provide similar lower limits of domain sizes (more than 240 molecules). Depending on the types of laser available to the experimenter, either the picosecond or microsecond laser technique in suitable for the estimation of photosynthetic domain sizes.

Acknowledgements

This work was supported by a National Science Foundation Grant (PCM-8308190) and, in part, by the Department of Energy (Contract AC02-76EV02386) at the Radiation and Solid State Laboratory. We thank Dr. M. Shahbaz for programming the computer.

References

1 Paillotin, G., Swenberg, C.E., Breton, J. and Geacintov, N.E. (1979) Biophys. J. 25, 513–553

2 Paillotin, G., Geacintov, N.E. and Breton, J. (1983) Biophys. J. 44, 65–77
3 Thornber, J.P., Markwell, J.P. and Reinman, S. (1979) Photochem. Photobiol. 29, 1205–1216
4 Geacintov, N.E., Breton, J., Swenberg, C.E. and Paillotin, G. (1977) Photochem. Photobiol. 26, 619–638; erratum: 29, 651–652
5 Geacintov, N.E. and Breton, J. (1982) in Advances in Laser Spectroscopy (Garetz, B.A. and Lombardi, J.R., eds.), pp. 213–237, Heyden & Son, Ltd., London
6 Van Grondelle, R., Hunter, N.C., Bakker, J.G.C. and Kramer, H.J.M. (1983) Biochim. Biophys. Acta 723, 30–36
7 Den Hollander, W.T.F., Bakker, J.G.C. and Van Grondelle, R. (1983) Biochim. Biophys. Acta 725, 492–507
8 Bakker, J.G.C., Van Grondelle, R. and Den Hollander, W.T.F. (1983) Biochim. Biophys. Acta 725, 508–513
9 Monger, T.G. and Parson, W.W. (1977) Biochim. Biophys. Acta 460, 393–407
10 Breton, J., Geacintov, N.E. and Swenberg, C.E. (1979) Biochim. Biophys. Acta 548, 616–635
11 Breton, J. and Geacintov, N.E. (1980) Biochim. Biophys. Acta 594, 1–32
12 Kramer, H. and Mathis, P. (1980) Biochim. Biophys. Acta 593, 319–329
13 Mauzerall, D. (1976) J. Phys. Chem. 80, 2306–2309
14 Breton, J., Roux, E. and Whitmarsh, J. (1975) Biochem. Biophys. Res. Commun. 64, 1274–1277
15 Thornber, J.P. (1975) Annu. Rev. Plant Physiol. 26, 127–158
16 Van Metter, R. (1977) Biochim. Biophys. Acta 462, 642–658
17 Burke, J.J., Ditto, C.L. and Arntzen, C.J. (1978) Arch. Biochem. Biophys. 187, 252–263
18 Mullet, J.E., Burke, J.J. and Arntzen, C.J. (1980) Plant Physiol. 65, 814–822
19 Brandimarte, S. (1982) Ph. D. Thesis, New York University
20 Paillotin, G., Geacintov, N.E. and Breton, J. (1983) Photochem. Photobiol. 37, 475–478
21 Geacintov, N.E. and Breton, J. (1982) in Trends in Photobiology (Helene, C., Charlier, M., Montenay-Garestier, T., and Laustriat, G., eds.), pp. 549–559, Plenum Press, New York
22 Geacintov, N.E., and Breton, J. (1982) in Biological Events Probed by Ultrafast Laser Spectroscopy (Alfano, R.R., ed.) pp. 157–191, Academic Press, New York
23 Seely, G.R. and Jensen, R.G. (1965) Spectrochim. Acta 21, 1835–1845
24 Thornber, P. (1980) Methods Enzymol. 69, 150–154
25 Shiozawa, (1980) Methods Enzymol. 69, 145–149
26 Schwartz, M., (1972) Methods Enzymol. 24, 139–146
27 Kan, K.S. and Thornber, J.P. (1976) Plant. Physiol. 57, 47–52
28 Shepanski, J.F., Williams, D.J. and Kalinsky, Y. (1984) Biochim. Biophys. Acta 766, 116–125
29 Sonneveld, A., Rademaker, H. and Duysens, L.N.M. (1980) Biochim. Biophys. Acta 593, 272–284
30 Marotti, P. and Lavorel, J. (1979) Photochem. Photobiol. 29, 1147–1151
31 Mathis, P., Butler, W.L. and Satoh, K. (1979) Photochem. Photobiol. 30, 603–614
32 Searle, G.F.W., Fraaije, J.G.E.M. and Schaafsma, T.J. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed., Vol. II, pp. 115–118, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
33 Bowers, P.G. and Porter, G. (1967) Proc. R. Soc. Lond. 296, 435–441
34 Yuen, M.J., Shipman, L.L., Katz, J.J. and Hindeman, J.C. (1980) Photochem. Photobiol. 32, 281–296
35 Lotshaw, W.T., Alberte, R.S. and Fleming, J.R. (1982) Biochim. Biophys. Acta 682, 75–85
36 Nordlund, T.M. and Knox, W.H. (1981) Biophys. J. 36, 193–201
37 Moya, I. and Tapie, P. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II. pp. 103–106. Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
38 Il'ina, M.D., Kotova, E.A. and Borisov, A. Yu. (1981) Biochim. Biophys. Acta 636, 193–200
39 Mullet, J.E. and Arntzen, C.J. (1980) Biochim. Biophys. Acta 589, 100–117
40 Moya, I., Mullet, J.E., Briantais, J.M. and García, K. (1981) in Photosynthesis I. Photophysical Processes – Membrane Energization (Akoyunoglou, G., ed.), pp. 163–172. Balaban International Science Services, Philadelphia, PA
41 Park, R.B. and Biggins, J. (1964) Science 144, 1009–1011
42 Beddard, G.S., Fleming, G.R., Porter, G., Searle, G.F.W. and Synowiec, J.A. (1979) Biochim. Biophys. Acta 545, 165–174
43 Shuvalov, V.A., Klevanik, A.V., Sharkov, A.V., Kryukov, P.G. and Ke, B. (1979) FEBS Lett. 107, 313–316