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Excitation energy annihilation in aggregates of chlorophyll *a*/*b* complexes

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Time-resolved picosecond absorption measurements were performed on aggregates of the light-harvesting chlorophyll (Chl) *a*/*b* complexes from spinach thylakoids. Variation of the intensity of the exciting laser pulse showed that efficient excitation annihilation occurs in these aggregates. From the time-integrated absorption decays, the domain size of these aggregates was calculated to be at least 300 and probably about 1000 chlorophyll *a* molecules. From the time-resolved chlorophyll *a* excited state decays, the rate of excitation annihilation per pair of excitations, γ_2, was calculated to be $(2–3) \cdot 10^9$ s^{-1}. It was shown that the annihilation competes with a 400 ps decay component in the mono-excitation decay, probably due to Chl *a*/*b* aggregates. A second decay phase of 2–3 ns is not associated with annihilation. The calculated domain size and annihilation rate constants correspond to a nearest-neighbour transfer rate of at least $2 \cdot 10^{11}$ s^{-1} but probably closer to 10^{12} s^{-1}. Our results show that although these Chl *a*/*b* complexes are dissociated from the membrane, their aggregation is such that fast energy transfer occurs over a large number of chlorophyll molecules.

Introduction

The excitation energy transfer dynamics in photosynthetic light-harvesting systems can be well investigated using high intensity picosecond flashes [1–4]. If, in a system consisting of connected antenna pigment molecules, a so-called 'domain', more than one excitation is generated, the annihilation of one or more of the excitations may occur upon collision. The efficiency of this process as indicated by the parameter γ_2, i.e., the rate of annihilation per pair of excitons in a domain, depends critically on the number of connected antenna molecules or the domain size, N_D, the rate of energy transfer between a pair of antenna molecules, k_h, and the efficiency of annihilation when a collision occurs. Usually, this latter parameter is chosen to be close to 1 [5,6]. Because annihilation is not the only process that occurs in a photosynthetic system, the rate of annihilation must be compared with the rate of single excitation decay, γ_1, that includes losses (fluorescence, triplet formation, internal conversion) and trapping. Usually, this competition is expressed by the

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Chl, chlorophyll; LHC, light-harvesting complex.

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parameter $r = 2\ \gamma_1/\gamma_2$ [1,2]. For large r values, trapping and losses dominate, for small r values, the annihilation is relatively strong.

So far, this phenomenon has been investigated mainly by measuring the time-integrated fluorescence yield as a function of laser energy. In general, one observes that the time-integrated fluorescence yield decreases if the laser energy is increased, and from the shape of the fluorescence yield vs. pulse intensity curve, combined with its position relative to the pulse intensity axis, the parameters N_D and r can be estimated. For chloroplasts with their reaction centers in the closed state PQ^-, γ_2 was estimated to be about $0.5 \cdot 10^9$ s^{-1} at room temperature, and a domain was thought to consist of at least 2–4 photosynthetic units [1,9]. With open traps, the situation in chloroplasts becomes very complicated. At relatively low pulse intensities, the yield of the fluorescence shows an anomalous enhancement [4,7,8]. Moreover, the trapping process seems to compete very efficiently with annihilations, contrary to what is expected [4,9]. An explanation for these phenomena may be found in a distinct partioning of the photosystem II (PS II) antenna in intact chloroplasts. On the other hand in photosynthetic purple bacteria, the fluorescence yield with open and closed traps and the trapping efficiency as a function of pulse intensity can be explained by the same set of parameters [5,10,11]. For instance, in *Rhodospirillum rubrum* and *Rhodobacter sphaeroides*, large domains are observed at room temperature ($N_D > 1000$), γ_2 is typically $5 \cdot 10^9$ s^{-1}, corresponding to $k_h = (1–2) \cdot 10^{12}$ s^{-1}. In isolated antenna systems, very similar parameters are obtained [12].

To study in more detail the excitation annihilation in chlorophyll-containing systems, we have measured the time dependence of the chlorophyll excited state as a function of the laser pulse intensity. These experiments were performed using large quasi-crystalline aggregates of the Chl *a/b* light-harvesting complex (LHC) [13]. Singlet-triplet annihilation experiments using similar aggregates have shown that energy transfer in these LHCs occur indeed very efficiently over a large number of chlorophyll molecules [14]. Although earlier streak-camera fluorescence measurements [15] suggested that excitation annihilation started only at intensities above 10^{15} photons/cm^2, our results demonstrate that, in fact, even at intensities of 10^{13} photons/cm^2, the excited state decay is not free of annihilation effects. The results show that efficient energy transfer takes place among at least 300 chlorophyll molecules, but probably more, with a nearest neighbour transfer rate of about 10^{12} s^{-1}.

Materials and Methods

Thylakoid membranes were isolated from spinach leaves in 0.4 M Sorbitol/0.1 M Tricine-NaOH (pH 7.8) and washed twice in the same medium. The isolation of the LHC II was performed using Triton X-100 solubilization of thylakoids followed by sucrose gradient centrifugation according to Burke et al. [16] as modified by Ryrie et al. [17]. The final LHC II preparation was suspended in 2 mM Tricine-NaOH, (pH 7.5). This preparation was dialyzed overnight against 5 mM Tricine-NaOH (pH 7.5)/5 mM EDTA/50 mM sucrose at +4 °C in the dark, immediately before use. In order to improve the optical properties of the sample and to obtain a more homogeneous LHC II aggregate distribution, 0.2% CHAPS was added and the sample was briefly sonicated.

The chlorophyll *a/b* ratio was typically about 1.1 and the main absorption maxima at 675 nm, due mainly to chlorophyll *a* and at 652 nm, predominantly from chlorophyll *b*. Picosecond experiments were performed using the pump-probe technique [18]. The light source was a cavity-dumped dye laser, synchronously pumped by a mode-locked argon ion laser. The maximum power at the sample was 4 mW at the excitation wavelength 678 nm with a pulse repetition rate of 800 kHz and a pulse width of 17 ps. The diameter of the spot at the point of overlap between the excitation and analyzing light beams was approx. 100 μm. The sample with an optical absorbance of 0.25 at 678 nm was filled into a rotating cell, 1 mm thick. To avoid accumulation of photoproducts in the beam, the cell was rotated at a speed high enough to ensure exchange of the excited sample volume between two laser pulses. The intensity of the excitation beam was changed by inserting neutral density filters ($A = 0.3–1.7$) in front of the sample cell.

The kinetic traces were fitted to the sum of three exponentials using a program based on the Marquardt algorithm [19].

Results

When Chl *a/b* light-harvesting complexes are excited by a picosecond laser flash, at 678 nm, a strong absorption decrease is observed upon probing at the same wavelength. The observed absorption changes are due to the generation of the chlorophyll *a* excited state [21]. A number of representative absorption recovery traces are shown in Fig. 1 at 678 nm. The traces correspond to one excitation per 18 Chl *a* molecules (a), one excitation per 90 Chl *a* (b), one excitation per 180 Chl *a* (c) and one excitation per 600 Chl *a* (d). The traces have all been normalized to their maximum value. It is very clear from the traces that the rate of decay of Chl *a** is increased dramatically when the pulse intensity is increased. This phenomenon is most probably due to excitation annihilation. In the remaining part of this paper, we shall try to give a consistent description of these results using the theory of excitation annihilation, as described by Paillotin et al [1] and Den Hollander et al. [2]. To apply these theories, it is necessary to construct a time-integrated fluorescence yield vs. pulse intensity curve. The former can be obtained by integrating the traces shown in Fig. 1 and normalizing with respect to the number of absorbed excitations. To do so, a fit of the experiment to a mathematical decay function is essential, especially for an estimate of the long-term behaviour of the decay. It happened that all decay curves fitted well with a three-exponential

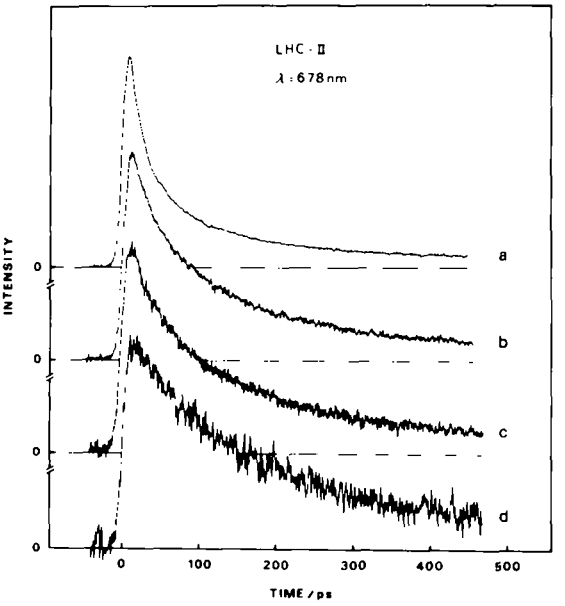

Fig. 1. Normalized absorption recovery kinetics at 678 nm of excited Chl *a/b* aggregates at different excitation intensities (number of absorbed photons/cm^2): (a) $2.5 \cdot 10^{14}$; (b) $5.0 \cdot 10^{13}$; (c) $2.5 \cdot 10^{13}$; (d) $0.8 \cdot 10^{13}$.

decay function. The parameter of these fits (lifetimes, amplitudes) are given in Table I, and again, one may observe the dramatic change in the decay parameters upon increasing the intensity. It should be noted that the component with the shortest lifetime (τ_1) has a very small amplitude at the lowest intensity of Table I. This also means that the error of this lifetime is large. However, because of its small amplitude, its contribution to the fluorescence yield is small. For the experiments with the highest pulse intensities, the decays probably did contain even faster components, but these are convoluted with the 17 ps laser pulse.

TABLE I

LIFETIMES (τ_i) AND RELATIVE AMPLITUDES (A_i) FOR THE Chl *a* EXCITED STATE RELAXATION AT 678 nm MEASURED AT DIFFERENT EXCITATION INTENSITIES

Absorbed photons/cm^2	Lifetime (ps)			Amplitude (%)		
	τ_1	τ_2	τ_3	A_1	A_2	A_3
$2.5 \cdot 10^{14}$	36	198	2500	63	32	5
$5.0 \cdot 10^{13}$	47	259	2500	37	56	8
$2.5 \cdot 10^{13}$	61	290	2500	32	59	9
$1.25 \cdot 10^{13}$	150	377	2500	44	41	15
$0.8 \cdot 10^{13}$	75	290	2500	3	80	17

The results could be consistently fitted assuming a long component of 2.5 ns. Fits with lifetimes of 3.0 and 2.0 ns were also performed, but these did not lead to significantly different results (i.e., within 20%) for the other components or for the integrated decays. The thus calculated time-integrated Chl a^* decays are shown in Fig. 2 and, as expected, the yields decrease strongly with increasing intensities. Although, this experiment is not accurate enough to estimate the true value of r, using the expression by Paillotin, a choice of $r \geq 3$ seems to give a reasonable fit to the data. This would correspond to $N_D \geq 1000$ Chl a molecules. Even if $r = 0$, N_D would still correspond to about 300 Chl a molecules, in reasonable agreement with the results obtained by Kolubayev et al. [14]. Assuming $r \geq 3$ it follows that $\gamma_2 < 2/3\gamma_1$.

From Table I, it is clear that even at the lowest pulse intensities, the decay of Chl a^* is not monoexponential, but that a large fraction of the decay of Chl a^* is due to a 300–400 ps component, while only the remaining 17% is due to the 2.5 ns decay. This suggests that $\gamma_2 < 3 \cdot 10^8$ s^{-1} or $\gamma_2 < 2 \cdot 10^9$ s^{-1}, depending on which value of γ_1 must be used. However, it is possible to calculate γ_2 also directly from the decays, especially from those where annihilation has just set in. If annihilation and losses determine the excitation decay,

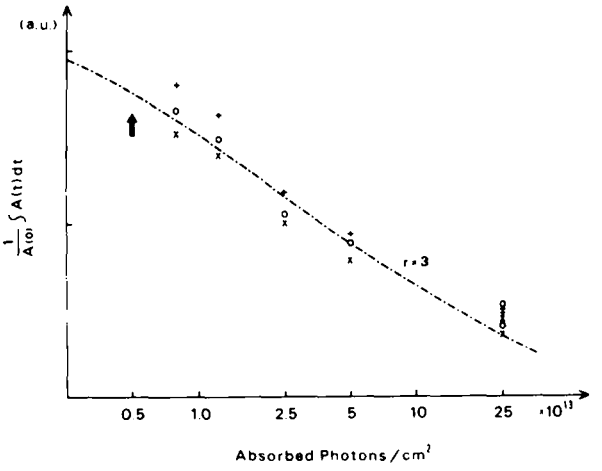

Fig. 2. Calculated normalized time-integrated absorption decays at 678 nm vs. number of absorbed photons/cm^2, with the long lifetime (τ_3) +, 3.0 ns, ○, 2.5 ns; ×, 2.0 ns. The dashed line represents a fit to eqn. 20 of Ref. 1 using $r = 3$. The point at which there is one excitation/domain is indicated by the arrow.

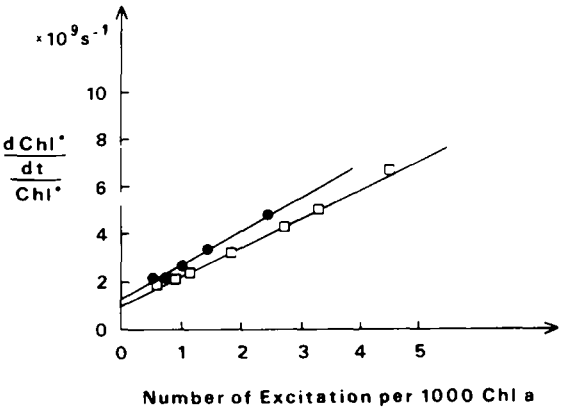

Fig. 3. This figure show (dChl a^*/dt)/Chl a^* as calculated from two different absorption recovery taces at 678 nm vs. number of excitations/1000 Chl a: ●, $1.25 \cdot 10^{13}$ photons/cm^2 and □, $2.5 \cdot 10^{13}$ photons/cm^2. The slope gives γ_2 as indicated in the text.

plotting (1/Chl a^*) dChl a^*/dt vs. Chl a^* should yield a straight line with slope, γ_2, according to the kinetic equation [1,2];

$$\frac{dn}{dt} = \gamma_1 n - \tfrac{1}{2}\gamma_2 \cdot n^2 \qquad (1)$$

where n stands for Chl a^*. The result of such a plot is illustrated in Fig. 3 for two experimental curves. In both cases, this plot results in the expected straight line, although the slope for the higher intensity curve is slightly smaller. The experiment indicated by ● yields $\gamma_2 = 2.8 \cdot 10^9$ s^{-1}, while the experiment indicated by □ yields $\gamma_2 = 2.4 \cdot 10^9$ s^{-1}. We believe that in this manner, a quite accurate estimate of γ_2 is obtained. The calculated values are rather close to γ_2, as estimated from the $r = 3$ curve, assuming $\gamma_1 = 3 \cdot 10^9$ s^{-1} ($\gamma_2 = 2.9 \cdot 10^9$ s^{-1}) and therefore support the assignment of this faster component to the main monoexcitation decay process, which is sensitive to excitation annihilation. If, in this fitting procedure, a smaller value of r had been used, larger values of γ_2 would have been obtained, but a preliminary calculation using $r = 1$ indicates that the general agreement is not as good.

Finally, using $\gamma_2 = 2.4 \cdot 10^9$ s^{-1} and $N_D = 1000$, we can calculate k_h, the pairwise energy transfer rate using Ref. 1;

$$\frac{\gamma_2}{2} = \frac{4k_h + \gamma_1}{N_D \cdot \phi_{N_D}}$$

where

$$\phi_{N_D} = \frac{1}{\pi} \ln N_D + 0.195$$

For $N_D = 1000$, we find that $k_h = 7.2 \cdot 10^{11}$ s^{-1}, and for $N_D = 300$, we find $k_h > 1.8 \cdot 10^{11}$ s^{-1}, which is the absolute lower limit. The kinetic traces obtained at higher excitation energies yield smaller values for γ_2 ($(1-2) \cdot 10^9$ s^{-1}). We believe that this effect is due to slight underestimation of the annihilation at the higher intensities, due to annihilation during the laser pulse. Although quantitation fluorescence yield measurements could have solved this problem, it is not likely to change significantly our conclusions.

Discussion

The transfer of excitations in the large aggregates of the Chl *a/b* complex occurs over a large number of chlorophyll molecules at a rate comparable to that observed in isolated antenna complexes of *Rb. sphaeroides* and *R. rubrum* [12]. Recently, the results from a study of singlet-triplet annihilation in Chl *a/b* LHC were interpreted in the same manner [14].

The efficient energy transfer in a large domain is manifested by the relatively efficient annihilation of excitations. The onset of annihilation occurs at an incident excitation intensity of 10^{13} photons/cm^2, similar to that observed for chloroplasts in the more recent experiments [1,8,9]. Also, the shape of the time-integrated normalized $\Delta A(t)$ is rather similar to that for the time-integrated fluorescence yield in chloroplasts. This indicates that the aggregation state of LHC II in detergent might be similar to that in the thylakoid membrane. Although attractive at first sight, one should be careful with such a conclusion. For the purple bacterium *Rb. sphaeroides*, it was recently concluded that the B800–850 antenna is essentially built from units of about 30–50 BChl 850s [10,11], although in the detergent LDAO, very large aggregates are observed ($N_D > 600$ BChl 850) [12]. The low intensity excited state decay for the Chl *a/b* LHCs is found to be biphasic. Even at the lowest intensities, where annihilation produces less than 10% quenching of the fluorescence yield, about 80% of the decay is with a lifetime of 0.3–0.4 ns, while 20% has a lifetime of 2.5 ns. Assuming that the 0.3–0.4 ns lifetime is slightly affected by annihilation, the true fast component is at most 0.4 ns. This must be compared with literature values 1.2 ± 0.5 ns (Nordlund and Knox [15]) and 0.65 and 0.33 ns (Ide et al. [21]). In fact, the weighted sum of the two latter values corresponds quite closely with our low intensity decay. It should be noted that the annihilation clearly competes with the 0.4 ns component, as judged from the value of γ_2 calculated from FIg. 3, suggesting that the 0.4 ns component corresponds to the higher aggregated Chl *a/b* complexes.

In conclusion, both the intensity dependence of the decay kinetics and the integrated time-course due to Chl *a** in Chl *a/b* LHC can be understood on the basis of the same physical parameters: $N_D = 1000$ Chl *a*, $\gamma_2 = 2 \cdot 10^9$ s^{-1}, or corresponding to a pairwise energy transfer rate of $k_h = 8 \cdot 10^{11}$ s^{-1}. A Chl *a** lifetime of about 3 ns is often found in isolated Chl *a/b* complexes [15,21,22] and, as mentioned above, a shorter lifetime is normally observed in large aggregates. In aggregated bacteriochlorophyll-containing light-harvesting complexes, one can often observe a scavenging process with a lifetime of 70–90 ps [24]. This excitation scavenger can be removed in some cases by addition of the reducing agent dithionite. It is possible that the 0.4 ns lifetime in Chl *a/b* aggregates is caused by a similar process. Our own work on Chl *a/b* complexes showed a 25 ps component of small amplitude when the complexes were excited at 665 nm. This process was attributed to excitation transfer from a blue-shifted Chl *a* chromophore (abs. max. approx. 670 nm [24]) to a red-shifted Chl *a* (abs. max. approx. 680 nm [24]). However, no such process was resolved in the present work (excitation at 678 nm), which is further support for our previous interpretation.

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