Picosecond Fluorescence Studies of Excitation Dynamics in Photosynthetic Light Harvesting Arrays

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Abstract.

The fluorescence decays of photosynthetic mutants of *Chlamydomonas reinhardtii* have been studied using picosecond resolution single photon counting. The mutants are used to reduce the complexity of the photosynthetic unit and enable assignment of the various decay components. The experiments are compared with analytical and numerical models for the energy transfer process leading to estimates for the single step transfer time (~200 fs) and number of visits before final trapping (2-4) in PSI arrays.
1. Introduction.

The light harvesting process, the process by which the energy absorbed by an array of chlorophyll molecules is transferred to the special chlorophyll molecules which perform the primary chemical act of photosynthesis, is quite phenomenally efficient. Once absorbed the energy from a photon has perhaps a 95% chance of being converted into chemical energy. Not merely is the efficiency of solar energy collection high in a static sense, but plants have evolved regulation mechanisms that respond to changes in light intensity and spectral distribution. The efficiency undoubtedly stems from the fact that the antenna molecules are held in proteins, with separations and perhaps orientations designed to facilitate energy transfer. Since the processes competing with transfer and final trapping of the energy (fluorescence and intersystem crossing) have relatively fixed time scales it is clear that the optimized light harvesting system will have very rapid energy transfer. From the physical point of view the details of the energy transfer and trapping are rather poorly understood at present. Understanding of the regulation mechanisms is even poorer, although a number of intriguing proposals exist (Bennett et al., 1980; Horton, 1983).

Measurement of the decay of excited state populations in the light harvesting arrays of chloroplasts holds considerable promise for the development of a comprehensive picture of the dynamics and regulation of energy flows in the light harvesting process. Picosecond time resolved fluorescence spectroscopy provides a sensitive and precise method of studying the population dynamics and has been exploited by several groups. The technique is sufficiently sensitive that very low light intensities may be used, thus avoiding the exciton annihilation artifacts which plagued earlier picosecond measurements. The form of the fluorescence decay curve is complex and recent studies have led to the conclusion that at least three exponential decay components
are necessary to fit the decay curve (Gulotty et al., 1982; Haehnel et al., 1982; Nairn et al., 1982; Karukstis and Sauer, 1984; Holzwarth, 1985). The relationship of these decay components to specific structural components has been rather speculative for a number of reasons. Firstly, the higher plant chloroplast has a complex constitution containing two reaction centers (PSI and PSII) one of which (PSII) may exist in two forms with differing properties, and a number of chlorophyll-protein complexes some of which are more closely associated with one type of reaction center than the other (Melis and Duysens, 1979). Thus decays may represent complex averages of the underlying processes. Secondly, it is not obvious that the decay of excitation in such a complex system should have exponential or sum of exponential form. For example, Pearlstein (1984) has pointed out that the fluorescence decay may be nonexponential if the excitation trapping is diffusion controlled rather than trap limited.

We have studied the fluorescence decay behavior of a series of photosynthetic mutants of *Chlamydomonas reinhardii* in order to simplify the observed decays and build up a physical description of the energy transfer dynamics (Gulotty et al., 1985a; Gulotty et al., 1985b; Gulotty and Fleming, 1985). The use of mutants has a major advantage over the standard biochemical isolation procedures, in preserving the integrity of light harvesting array, and enables a definitive assignment of the components observed in the wild-type decay. After a brief discussion of our experimental methods, the remainder of the paper is devoted to a discussion of three major topics: (a) the origin of the decay components of the wild-type chloroplast, (b) the dynamics of energy transfer in the PSI core chlorophyll-protein and (c) the dynamics of energy transfer in the PSII-lacking Chl a/b protein deficient mutant A4-d.
II. Experimental Methods.

The *C. reinhardtii* wild type and mutant strains were grown heterotrophically in low light on tris-acetate/phosphate buffer. The biochemical characterization of the various mutants is described elsewhere (Gulotty et al., 1985).

The time resolved fluorescence decay measurements were carried out using the time correlated single photon counting method. A review article describing our apparatus will appear elsewhere (Chang et al., 1985), and only brief details are given here. A diagram of the apparatus is shown in Figure 1. The light source was a synchronously pumped, cavity dumped dye laser generating 10 ps pulses at 75 kHz repetition rate. The laser wavelength was generally kept at 610 nm. Fluorescence was detected by a Hamamatsu R1645-01u microchannel plate photomultiplier amplified by a Hewlett-Packard PH 8447f amplifier. Fluorescence was collected at 90° from the flowing sample through a Ditric Optics 3 plate filter at 680 nm. Dark adapted conditions were maintained by flowing the sample at 1ℓ/min. The instrument response function of the single photon counting apparatus was measured by scattering from a suspension of non-dairy creamer in water placed in the flow-cell. The instrument function had a FWHM of 130 ps and a full width at tenth maximum of 250 ps. For a single exponential decay the time resolution of the apparatus is 20-30 ps.

The data were analyzed as sums of exponentials by the standard method of iterative convolution. Single exponential standards of appropriate lifetime were run before and after each set of measurements. As discussed by Cross and Fleming (1984) and Chang et al. (1985) the single photon counting method can give data in which only statistical (counting) error is detectable. This does not, of course, mean that arbitrary numbers of parameters may be extracted from a decay curve. In simulations in which four exponential components were
convoluted with a measured instrument function and appropriate Gaussian noise added, it was found that for $10^4$ peak counts only three components could be reliably recovered, whereas for $10^5$ peak counts the input parameters were accurately extracted from the fit. In other words the "information content" of the $10^4$ peak count curve is insufficient to enable extraction of four decay times and four pre-exponential factors.
III. Results and Discussion.

(a) Origin of the Decay Components in the Wild-Type Chloroplast.

Figure 2 shows typical single photon counting fluorescence decay data for the *C. reinhardtii* wild-type strain 2137 (curve a), PSI mutant strain 12-7 (curve b), PSII mutant strain 8-36c (curve c) and the PSI-PSII mutant strain C2 (curve d). (Note that PSI mutant refers to a mutant lacking PSI i.e. containing PSII). The figure illustrates the nonexponential decay kinetics of the strains 2137, 12-7 and 8-36c, all of which have reaction centers and the nearly single exponential behavior of the PSI-PSII mutant C2. The PSI and PSII mutant strains show an increased weight of long lifetime component compared with the wild-type. This component has a similar lifetime to the C2 mutant which lacks both reaction centers, i.e. there are excitations with "nowhere to go" in both the PSI and PSII mutants. The mutant lacking PSII has a much larger weight of this component as might be expected since most of the Chl a/b protein in the wild-type is presumed to be connected to PSII. The short time behavior of the PSI mutant (τ₁ = 155 ps) shows a longer decay than both the wild-type (τ₁ = 89 ps) and the PSII mutant (τ₁ = 53 ps).

Figure 3 shows a typical fluorescence decay of the wild-type strain 2137 of *C. reinhardtii*. The smooth curve is the best fit of the data, f(t), using a sum of three exponential components convoluted with the instrument function. The residuals shown above the decay curve show that a satisfactory fit is only achieved when three components are used. This was also true for the PSI and PSII mutants and the data are collected in Table I.

At this point it is important to address the question: Is the true fluorescence decay really a triple exponential or is it a different (for example more complex) function that due to the limitations of real data (e.g. finite time resolution, dynamic range, etc.) can be statistically well fit as a triple exponential decay? We address this question in two stages: we first
describe a simulation of wild-type decay from the decays of the three mutants; second, a discussion of the use of sums of exponentials is given in part (c) of this discussion.

Figure 4 summarizes the results of a simulation of the wild-type fluorescence in which we sum the decay properties of the PSI and PSII mutants which correspond to the photochemical paths and use the average lifetime of the PSI-PSII mutant to represent the lifetime of decoupled Chl a/b protein. The curves in Figure 4 were constructed according to

\[ f(t) = \alpha f(t)_{\text{PSI}} + \beta f(t)_{\text{PSII}} + \gamma f(t)_{\text{decoupled antenna}} \]  

(1)

The simulated curves were convoluted with a real instrument function, had Gaussian noise added (not shown in Figure 4 for clarity) and fit as if they were experimental results. Curves (a) and (c) illustrate the range of the simulation, whereas curve (b) follows the real data very closely. Curve (b) was constructed with \( \alpha = 0.6, \beta = 0.3925 \) and \( \gamma = 0.075 \). While all values of \( \alpha, \beta \) and \( \gamma \) resulted in curves that could be fit to three exponential decay components (despite the four input components) with a \( \chi^2 \) of 1.0-1.2 (for \( 10^4 \) peak counts), only the convolutions with approximately equal weights of PSI and PSII fit the wild-type data. Since the intermediate lifetime in both mutants is 424 ps (Table 1), the major conclusion of the simulation is that the short component lifetime of 89 ps in the wild-type *C. reinhardii* is an average of a 53 ps due to energy transfer and trapping at PSI and a 152 ps lifetime to energy transfer trapping at PSII.

(b) Excitation Dynamics in PSI.

We can use the 53 ps component assigned to energy transfer and trapping within the core antenna of PSI, in combination with biochemical and spectral
data to provide estimates of two key quantities: the single step transfer time between communicating Chl molecules and the number of visits made to the reaction center before final trapping occurs. The starting point is Pearlstein's (1982) expression for the excitation lifetime, $M_0$.

$$M_0 = [1+(F_d/F_t)(N-1)]k_p^{-1} + [(qF_t)^{-1} - (qF_a)^{-1}][(N-1)^2/N] + aNF_a^{-1}$$  \hspace{1cm} (2)

Here $F_t$ and $F_d$ are the average Forster rate constants (forward and reverse) for excitation transfer between antenna chlorophyll and the reaction center, $F_a$ is the average Forster rate constant for excitation transfer between antenna molecules, $N$ the array size, and $k_p$ is the primary electron transfer rate constant in the reaction center. The parameter $q$ is the coordination number of the lattice and $a$ is a numerical constant that depends on the array type (Hemenger et al., 1972). An important feature of eqn. 2 is that it accounts for multiple visits to the reaction center without requiring any assumption about the actual number of visits.

Equation 2 contains a number of parameters of which only $M_0$ and $N$ are accessible from our data. However, enough spectral and kinetic data are available to make reasonable estimates of $F_d/F_t$, $F_t/F_a$ and $k_p$. In our calculation we closely follow the assumptions used by Shipman (1980). We assume $F_d/F_t$ is 0.107 which is simply the Boltzmann factor between a 700 nm trap and the average antenna absorption of 678 nm. Assuming the P700 is a dimer and using the same Boltzmann factor we set $F_t/F_a = 1.56$. The value for $k_p = (3 \text{ ps})^{-1}$ is based on the measurements of Parson et al. (1980) for the rate of primary electron transfer in a bacterial reaction center. Our calculation shows that single step transfer times are quite insensitive to the value of $k_p$ in PSI systems. From our data for the PSII mutant 8-36C we let $M_0 = \tau_1 = 53 \text{ ps}$ and $N = 111$. The remaining parameters in (2) are $q$ and $\alpha$. We carried
out calculations for both a square and a simple cubic lattice for which q = 4, \( \alpha = 0.429 \) and q = 6, \( \alpha = 0.262 \) respectively (Hemenger, 1972).

Putting all the above parameters into equation 2 leads to a single step transfer time \( (1/qF_a) \) of approximately 0.1 ps for both lattice types. The calculation yields the trapping and detrapping times \( 1/qF_t \approx 70 \) fs and \( 1/qF_d \approx 0.6 \) ps for both lattice types. If the short lifetime is due only to PSI core Chl a molecules, then \( N = 40-60 \) (Vierling and Alberte, 1983) and the single step transfer time is 0.4 - 0.7 ps.

The second quantity of interest is the ratio of the lifetime \( M_0 \) to the time of first passage \( \tau_{FPT} \) (i.e. the time between excitation and first arrival at the reaction center. If \( M_0/\tau_{FPT} \) is 1, the photochemical event is "diffusion controlled." If \( M_0/\tau_{FPT} > 1 \) multiple visits occur and the photochemical event is reaction controlled. \( \tau_{FPT} \) is calculated from Pearlstein (1982).

\[
\tau_{FPT} = N/qF_t + [\alpha - (1/q)]N/F_a
\]  

Using the values calculated above in (3) with \( N = 111 \) we find \( M_0/\tau_{FPT} = 3.7 \) for both the square and cubic lattices and conclude that the excitation makes about four visits to the reaction center before the photochemical event occurs. However, if we assume \( N = 40-60 \) then \( M_0/\tau_{FPT} = 1.4-1.7 \) and the kinetics are closer to diffusion controlled.

(c) The A4-d mutant - comparison of the fluorescence decay curve with a simple model.

The _C. reinhardtii_ strain A4-d mutant is produced by crossing the PSII mutant Bl with a Chl a/b protein deficient strain DS521 (Gulotty, 1985). A4-d has 80-110 Chl/P700 and a Chl a/Chl b ratio of 4-5:1. The fluorescence decay (see Table 1) contains significantly less long component than the PSII mutants (such as 83-C or Bl) that contain substantial amounts of Chla/b protein.
Despite the significantly reduced complexity the decay still remains nonexponential.

We have attempted to model the excitation dynamics in A4-d and to address the sum of exponential fitting of the observed decays by considering the behavior of excitations in cylindrical arrays of chlorophyll molecules. The method of calculation, described in detail elsewhere (Gulotty and Fleming, 1985), involves numerical integration of the relevant master equations (Shipman, 1980). In the previous section we suggested that even in PSI core (and certainly in PSII core) trapping was reaction controlled. We have previously suggested that the longer lifetime components in PSI particles or PSII-containing mutants arise from Chl molecules not well coupled to the PSI core. In order to investigate this we have studied the excitation dynamics in two coparallel cylinders, one of which contains a reaction center and one of which contains only Chla/b protein. The use of the separate cylinder model for the light harvesting array of higher plants is motivated by analogy with the bacterial light harvesting structure (Miller, 1982) and by electron microscopy data on higher plants in which the light harvesting array appears to increase in units of $30^2 \text{Å}^2$ from $90-180^3 \text{Å}^3$ (Staehelin and DeWit, 1984). The model may be used to discuss the changing association of light harvesting Chl proteins with PSI and PSII due to photophosphorylation (Bennett et al, 1980), by changing the intercylinder distance. In addition the transfer of excitations between Chl in different thylakoid membranes, as in grana stacks, may be modeled in this way.

The A4-d system was modeled by means of two spatially separated cylinders containing randomly placed chlorophyll molecules. One cylinder contains a trap whereas the other contains only light harvesting Chl. The total number of molecules (90) and the spectral forms where chosen to model the Chl content
of the A4-d. A cylinder 50Å high with a radius of 36Å containing a trap at 700 nm and 59 Chla molecules (678 nm) was chosen so that the Chl density was 3400Å³/Chl. A coparallel cylinder contained 30 molecules (15 Chla, 671 nm and 15 Chlb, 653 nm) had the same height but a radius of 25.5Å in order to keep the density the same as in the first cylinder. This model is consistent with the structure of the bacterial reaction center and light harvesting BCHl as determined by electron microscopy by Miller (1982) in which six smaller discs containing light harvesting BCHl surround a larger disc containing a bacterial reaction center. In placing the Chl molecules a constraint of \( R_{ij} > 12 \AA \) was used to avoid concentration quenching (Beddard and Porter, 1976). The two cylinders were separated by 20Å.

Figure 5 shows the result of the numerical integration of the master equations for two cases (a) where the trap is P700 and (b) where the trap is P680. Both curves fit perfectly to a double exponential decay. The parameters are listed in Table 1. Comparison shows that the short component in PSII has about a three-fold longer lifetime. This ratio is similar to the experimental values deduced by Gulotty et al. (1985) and Wendler et al. (1985). The factor of three between PSI and PSII has two sources in our calculation. The major contribution (about a factor of two) comes from the difference in trap depth. The remaining contribution comes from our assumption of a dimeric trap in PSI and a monomeric trap in PSII. Before our calculation is accepted as support for monomeric PSII and dimeric PSII traps it must be noted that the core array size for PSII is not well-known, and there may well be a difference in the primary electron transfer rate in the two centers (here assumed = (3 ps)⁻¹ in both). Our calculation suggests that the decay time of PSI is more sensitive to the monomer/dimer question whereas the PSII decay is more sensitive to the electron transfer rate. This reflects the higher probability of detrapping in PSII.
Curve (a) is very similar to that of the A4-d mutant, in both lifetimes and weights of the components. The agreement in lifetime is largely due to the choice of Chl density whereas the agreement in relative weights is due to the choice of intercylinder distance. The decomposition of the mutant decay into a short (50 ps) component originating from excitation of core Chla molecules and a longer (425 ps) component originating from excitation of a light harvesting array relatively poorly coupled to the PSI core is supported by the decomposition shown in Figure 6. Curve (a) is the population decay when the reaction center is excited directly. Curve (b) shows the decay when only the 678 nm molecules (core Chla) are excited directly. A 50 ps single exponential decay is observed. Curves (c) and (d), which are almost indistinguishable, give the decay when the 671 nm or 653 nm Chl are directly excited. In this case the population decays nonexponentially for about 200 ps and then decays exponentially with a time constant of about 300 ps. The slow initial decay in the light harvesting cylinder is expected since it will take some time for the excitation to find the trap-containing cylinder. It is somewhat surprising, perhaps, that when all the Chl molecules have equal excitation probability the curves a-d in Figure 6 give the perfect double exponential of Figure 5 curve (a). Thus our calculation strongly supports a multiple exponential fitting of experimental decay curves.
IV. Conclusions.

Photosynthetic mutants appear to be very valuable for unraveling the complexity of the light harvesting mechanism in green plants. Our study of C. reinhardtii mutants has led us to conclude that (1) fluorescence associated with the presence of PSI reaction centers must be included in analyses of wild-type chloroplast decays. (2) The true wild-type decay is considerably more complex than the sum of three exponential components. Thus an attempt to model photophysical behavior in chloroplasts in terms of three components is an unacceptable oversimplification. (3) Calculations suggest a single step transfer time in the PSI core to be about 200 fs. We also find that an excitation makes between 2-4 visits to PSI before final trapping. (4) Numerical simulations justify a multi-exponential analysis and suggest the threefold difference between PSI and PSII lifetime largely arises from the greater detrapping probability in the latter.

Acknowledgements

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References.


Table I. Fluorescence decay parameters of *C. reinhardtii* wild-type and mutant strains.

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<tr>
<th>Sample Type</th>
<th>Strain</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$\tau_3$</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_3$</th>
<th>$x^2_R$</th>
<th>Time Scale (ps/channel)</th>
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* Fluorescence emission collected at 680±5nm, excitation 610 nm.

* No noise in calculated curves.
Figure captions.

Figure 1.

Experimental arrangement for time-correlated single-photon counting. MCP: multichannel plate; CFD: constant fraction discriminator; TAC: time-to-amplitude converter. The case of ultraviolet excitation is shown above. If the pulses from the cavity dumper are vertically polarized, a \( \lambda/2 \) plate must be inserted in the laser beam before the nonlinear crystal (usually LiIO\(_3\) or KDP) in order to produce vertically polarized UV. The polarizer, \( P_1 \), "cleans up" the polarization of the excitation beam. \( F \) is either an interference or cutoff filter to select a portion of the fluorescence spectrum and to discriminate against scattered light. \( P_2 \) is a polarizer whose transmission axis is set either parallel, perpendicular, or at 54.7\(^\circ\) to the polarization of the excitation.

Figure 2.

Fluorescence decay curves of \textit{C. reinhardii} strains (a) wild-type WT2137 (b) PSI mutant 12-7 (c) PSII mutant 8-36C and (d) PSI-PSII mutant C2. The excitation wavelength is 610 nm and emission was collected at 680 \( \pm \) 5 nm.

Figure 3.

Fluorescence decay curve of wild-type \textit{C. reinhardii} strain WT2137. \( f(t) \) (dots) instrument response function \( i(t) \) (dots) and fit of the data \( f(t) \) (curve) with a sum of three exponential decay components, \( 0.544 \exp(-t/95 \text{ ps}) + 0.439 \exp(-t/371 \text{ ps}) + 0.018 \exp(-t/2277 \text{ ps}) \). Shown above are normalized residuals as in eq. 2 for fits of the data \( f(t) \) (dots) to (a) three, (b) two, and (c) one exponential decay components. The residuals (d) are calculated for a single exponential fit to oxazine 725 in water where the \( \chi^2_r \) is 1.0 and the lifetime is 490 ps. The excitation wavelength is 610 nm, and the emission was collected at 680 \( \pm \) 5 nm.
Figure 4.

Simulation of *C. reinhardtii* wild-type fluorescence decay data.
(a) $f(t) = 0.9925$ PSII mutant decay + 0.0075 PSI-PSII mutant decay, (b) (curve)
$f(t) = 0.6$ PSII mutant decay + 0.3925 PSI mutant decay + 0.0075 PSI-PSII
mutant decay, (b) (dots) $f(t) =$ wild-type decay, (c) $f(t) = 0.9925$ PSI mutant
decay + 0.0075 PSI-PSII mutant decay. $f(t)$ was convoluted with typical
instrument response function to yield the curves and dots (a)-(c).

Figure 5.

Population decay calculated for equal excitation probability of chloro-
phyll molecules in two separate cylinders, one of which contains a PSI trap
(curve a) or a PSII trap (curve b) and the other contains Chl a/b protein with
no trap. The intercylinder separation is 20 Å.

Figure 6.

Excited state population decay of spectrally heterogeneous array in
separate cylinders (inter-cylinder distance is 20 Å, 3400 Å³/Chl) with
different initial excited state population distributions. (a) P700 reaction
center only, (b) Chl a 678 nm only, (c) Chl a 671 nm only, and (d) Chl b
653 nm only.
Figure 3.
Figure 4.
Figure 5.

![Graph showing the relationship between excited state population and time.](image-url)
Figure 6.