Modification of room-temperature picosecond chlorophyll fluorescence kinetics in green algae by photosystem II trap closure

I. Moya, M. Hodges and J.-C. Barbet

Laboratoire de Photosynthèse du CNRS, 91190 Gif-sur-Yvette, France

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Room-temperature single-photon timing measurements on green algae at low excitation energies indicate at least three kinetic components of chlorophyll fluorescence when the reaction centres of photosystem (PS) II are open (F_0) with lifetimes of approx. 50 ps (fast), 200 ps (middle) and 450 ps (slow). The absence, in green algae, of a long-lifetime component (1.4 ns) is at variance with previous reports. Closing the reaction centres gave rise to a similar increase in both the middle and slow lifetimes, by a factor of between 4 and 5, to produce lifetimes of approx. 850 ps and 2 ns, respectively, when all traps were closed (F_{max}). The yield and lifetime of the fast component were found to be independent of PS II centre closure. The yields of the middle and slow components were both modified by reduction of the primary stable electron acceptor of PS II (Q_A) with the slow-component yield increasing by a greater degree than the middle-component yield. It was also observed that as PS II reaction centres were closed to photochemistry the fluorescence yield and average lifetime increased proportionally. This trend and the similar increases in the middle and slow lifetimes are indicative of a well-connected system of PS II units favouring exciton transfer between PS II centres. We suggest that the fast kinetic component arises mainly from PS I while the middle and slow components arise from different types of fluorescing unit located in the granal lamellae and associated with a system of well-connected PS II.

Photon counting *Chlorophyll fluorescence lifetime* *Photosystem II* *Photosynthesis* *Photosystem I* *Exciton transfer*

1. INTRODUCTION

Recent studies concerning the lifetime of chlorophyll fluorescence after picosecond pulse excitation of intact green algae and higher plant chloroplasts have revealed multiexponential decay kinetics that indicate a sum of at least 3 components spanning a time range from 0.05 to 2.2 ns [1–3]. These have often been referred to as the fast (50–150 ps), middle (400–750 ps) and slow (1–2.2 ns) components. They undergo complex changes in lifetime and yield as the PS II reaction centres are closed. For instance, it has been shown that with mainly open reaction centres the decay exhibited by *Chlorella pyrenoidosa* is dominated by two components with lifetimes of 100 ps (18% yield) and 500 ps (78% yield) with only a small contribution from a slow component (1.4 ns, 4% yield). Closing the reaction centres increased the yield of the long-lived decay from 4 to 65%, at F_{max}, while increasing its lifetime from 1.4 to 2.2 ns [2]. Such changes were interpreted in terms of the preence of only a limited degree of energy transfer between PS II reaction centres. The extent of the change in lifetime of the slow component between F_0 and F_{max} has been consistently reported

Abbreviations: PS, photosystem; LHC, light-harvesting Chl *a*/*b*-protein complex; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Q_A, primary stable electron acceptor of PS II; F_{max} (F_0), maximum (minimum) chlorophyll fluorescence

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to be approx. 2-fold [2,4–7] while at F_0 the lifetime of the slow component appears to be species-dependent [6]. The lifetime of the fast component remains relatively unaffected by PS II trap closure while that of the middle component increases with its yield increasing to a lesser degree (see e.g. [2]).

It has been proposed that the slow phase could originate from PS II radical pair recombination between $P680^+$ and reduced pheophytin. This follows the idea put forward by Klimov et al. [8] that variable fluorescence is in fact luminescence. This was concluded from the appearance of a 4 ns fluorescence delay component when Q_A was chemically reduced prior to illumination which followed the disappearnce of reduced pheophytin. The long-lived component could arise directly from the recombination process or re-excited antenna pigments (see [6]). However, charge recombination is not essential to produce a long-lived decay as such a component is still seen in a mutant lacking PS II [7]. Furthermore, the opposite dependence of the yield of the slow and fast components on closing of PS II is difficult to explain by such a process [2]. The two faster components have been ascribed to excitons lost prior to reaching the reaction centres, the fast component arising from PS II core chlorophyll and the middle component from the LHC [1,4].

An alternative hypothesis for the origin of the 3 components has been put forward by Butler et al. [9]. By re-examining the earlier data of Haehnel et al. [2] in terms of α/β PS II heterogeneity [10], they suggested that the middle component arose from PS II_β (isolated units with a small antenna) and the slow phase from closed PS II_α (connected units with a large antenna). The fast decay was attributed to both open PS II_α centres and to PS I. Although the idea of α/β heterogeneity has been difficult to prove by redox titrations of chlorophyll fluorescence [11], the contribution by PS I to the fast-lifetime component has been substantiated by several groups [12,13] and has also been inferred from studies of mutants [6,7]. Studies of photosynthetic mutants [6,7] and chloroplasts at different stages of development [5] have been useful in showing that the overall decay is probably more complex than the tri-exponential model used and that perhaps each of the 3 components has multiple origins.

A striking point is that, in spite of the heterogeneous nature, the overall decay gives rise to an average lifetime, calculated from the deconvoluted parameters, which shows an almost proportional increase with total fluorescence yield by a factor of 3–5 [2]. Such a relationship was found previously using phase fluorimetry [14,15] and was interpreted as corresponding to a well-connected organisation of PS II favouring energy transfer between centres in agreement with the proposals of Joliot and Joliot [16].

Here, we describe the results of the fluorescence decay decomposition obtained with an improved single-photon fluorimeter having an instrumental response function of 60 ps. Special attention has been paid to producing homogeneous sample illumination and analysing well-defined fluorescence levels. The results are discussed in terms of a heterogeneous pool of well-connected PS II reaction centres.

2. MATERIALS AND METHODS

Chl. pyrenoidosa and *Chlamydomonas reinhardtii* were grown as in [17] and diluted to 20 μg Chl/ml for experimentation. Fluorescence measurements were carried out at 90° to the excitation beam in a 2 × 2 mm cuvette located at the entrance slit of a monochromator of 3 nm bandwidth. Flow of the algal suspension (400–500 ml) was by means of a peristaltic pump at up to 20 ml/s. The very small dimensions of the cuvette provide several advantages, including homogeneous sample excitation, and allow us to obtain a flow rate of 500 cm/s (i.e. the sample is illuminated for only 200 μs at maximum pumping speed). The low light intensity (2×10^7 photons per pulse) in addition to the high flow rate allowed us to obtain an 'accurate' F_0 measurement. The F_{max} level was obtained after incubation with 20 μM DCMU using stationary samples, the analysed sample being changed every 10 s during the several minutes required to complete the measurement. The intermediary fluorescence levels were obtained from either dark-adapted or preilluminated algae (white light, 5 $W \cdot cm^{-2}$) at different flow rates. The intensity of the laser was kept non-actinic during these procedures.

The single-photon timing apparatus employed in these studies consisted of a mode-locked and cavity dumped dye laser system (Spectra-Physics; dye

DCM) synchronously pumped by an Ar^+ laser. This system is wavelength-tunable in the range 610–700 nm and provides pulses of 10–15 ps duration at a repetition rate of up to 4 MHz. Because the repetition rate of the excitation pulses was about 1000-times greater than the rate of the fluorescence events, the reverse configuration was adopted. The start input to the time-to-amplitude converter (TAC) was produced by the fluorescence while part of the excitation beam was directed to a fast photodiode (rise time < 1 ns) which provided the stop pulse. Fluorescence photons were detected by a microchannel plate photomultiplifer (Hamamatsu R1564U), having an S 20 spectral response, and the output pulses were passed to a constant fraction discriminator through a 24 db gain wide-band amplifier (1 GHz). Under these conditions the instrumental response function was approx. 60 ps FWHM (full-width at half-maximum) when scattered light was examined instead of fluorescence. The experimental excitation function and the fluorescence decay were consecutively recorded in a 512-channel memory group of a multichannel analyser (Tracor Northern 1750) and transferred to a Hewlett Packard 9836 computer. Deconvolution of the experimental decays into a sum of exponentials was carried out by means of a least-squares programme using the Marquardt search algorithm for the non-linear parameters. The quality of the fits was judged by the reduced χ^2 criterion and the plot of the weighted residuals.

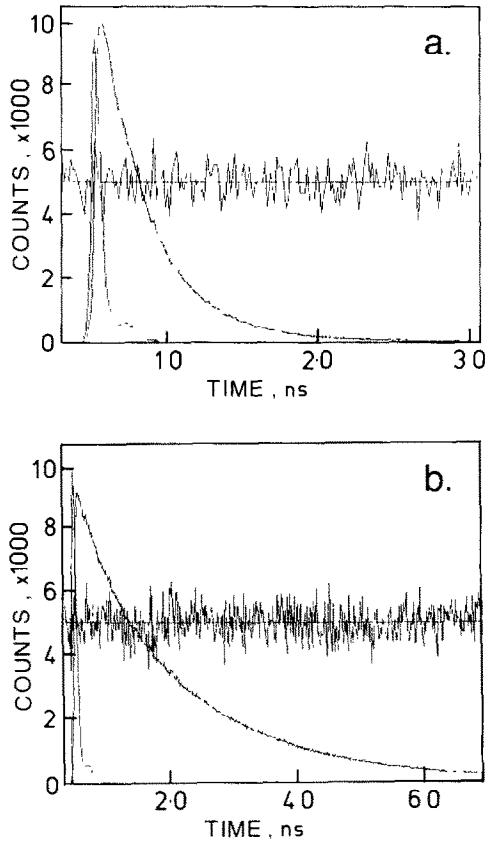

Fig.1. Chlorophyll fluorescence decays of *Chl. pyrenoidosa* at 685 nm at (a) F_0 (Q_A oxidised) and (b) F_{max} (Q_A reduced). The excitation profile shown on the plot has a width of approx. 60 ps (FWHM). The best fit of a 3-exponential decay model is superimposed with the experimental fluorescence decay. The weighted difference between the two curves is shown in the centre of the plot (scale +10 to −10).

3. RESULTS

Fig.1 shows the instrumental response function to scattered light and the chlorophyll fluorescence decay of *Chl. pyrenoidosa* measured at 685 nm following excitation at 625 nm at the F_0 (fig.1a) and F_{max} levels (fig.1b). Three exponentials were required to fit the data at both the minimum and maximum fluorescence levels (see also table 1). When all Q_A^- was oxidized (F_0) the 3 components had lifetimes of 40 (fast), 200 (middle) and 454 ps (slow) (see fig.1a). Similar values were found with the same batch of algae measured several times during the same day as well as from experiments carried out on different days. No component having a lifetime slower than 0.5 ns was found in *Chlorella*, at variance with previous results concerning green algae [1,2]. However, similar lifetimes at F_0 have been reported for barley chloroplasts suspended in the presence of Mg^{2+} [6]. Addition of DCMU, at the F_0 level, induced a 20% increase in total fluorescence yield which specifically affected the middle and slow component lifetimes. At F_{max} (fig.1b) the 3 components exhibited lifetimes of 70 ps (fast), 700 ps (middle) and 1.90 ns (slow) which are in good agreement with the data, concerning green algae, of other groups [1,2]. Several intermediary levels of Q_A reduction were also analysed. Good fits always required at least 3 exponentials to obtain χ^2 values of approx. 1.0, indicating that the residuals cor-

responded to Poissonian noise. Two components introduced systematic deviations in the weighted residuals and a subsequent increase in the χ^2 value, while 4 components only decreased the χ^2 values by less than 0.1 in many cases.

Fig.2 shows the relationship between average fluorescence lifetime and measured total chlorophyll fluorescence yield for *Chl. pyrenoidosa*. The linear relationship confirms previous data obtained by phase fluorimetry [14,15] and by single-photon counting [1]. This means that the plots shown in fig.3 can represent the change in the 3 components as a function of total fluorescence yield.

Fig.3a shows the evolution of the lifetimes of each component as PS II reaction centres are closed (as indicated by the average lifetime). The proportional increase in both the slow (from 0.45 to 2.0 ns) and middle (from 0.18 to 0.85 ns) components and the 4–5-fold increase in the lifetimes of these two components on increasing the number of closed PS II centres between F_0 and F_{max} are striking observations. The lifetime of the fast component stayed relatively unchanged (60–100 ps).

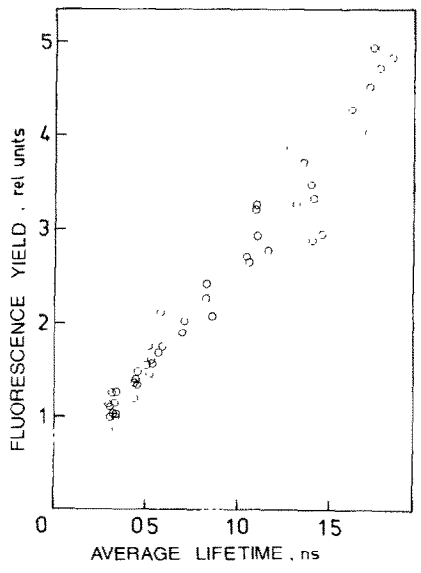

Fig.2. Total chlorophyll fluorescence yield of *Chl. pyrenoidosa* as a function of average lifetime calculated using the equation $\tau_{mean} = \sum_{i=1}^{3} \alpha_i \tau_i^2 / \sum_{i=1}^{3} \alpha_i \tau_i$, where α_i is the amplitude and τ_i the lifetime of the i-th component, and the values obtained from the best 3-exponential fit to the decay.

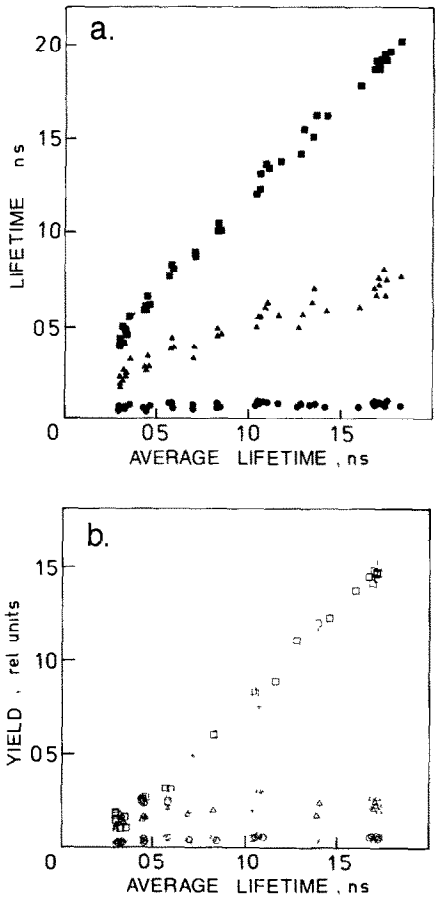

Fig.3. The effect of PS II trap closure, as monitored by the average fluorescence lifetime, on (a) the lifetime (closed symbols) and (b) yield (open symbols) of the slow (square), middle (triangle) and fast (circle) components of the fluorescence decay kinetics of *Chl. pyrenoidosa*.

These experiments have been repeated a number of times and have always yielded the same trend and similar large changes in lifetimes to those depicted in fig.3a.

Fig.3b shows the change in yield of each component as the average lifetime is altered. As for the corresponding lifetime changes (fig.3a) only the slow and middle components are altered by changing the state of the PS II reaction centres, whereas the yield of the fast component remains independent of PS II trap closure. This latter observation is at variance with those in [2] in which the fast component decreased on closing PS II traps. However, the exact relationship between the yield of the slow and middle components and the total

Table 1

Decay components of the chlorophyll fluorescence kinetics in *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii*[a]

		Lifetimes (ns)			Yield (relative units)			Average lifetime, τ_{mean} (ns)
		τ_f	τ_m	τ_s	ϕ_f	ϕ_m	ϕ_s	
Chl. pyrenoidosa	F_0	0.050	0.190	0.415	0.02	0.12	0.16	0.301
	F_{max}	0.090	0.740	1.945	0.04	0.22	1.49	1.751
C. reinhardtii	F_0	0.060	0.242	0.545	0.04	0.14	0.22	0.390
	F_{max}	0.095	0.945	2.278	0.03	0.33	1.67	2.029

[a] All data were calculated on the basis of a 3-exponential decay model. Subscripts f, m and s refer to the fast, middle and slow components, respectively

fluorescence yield is complicated by occasional variations in day to day experiments. The main trends and extents of the changes are depicted in fig.3b in which the yield of the middle and slow components did not increase proportionally with their respective lifetimes (it should be noted that on several occasions a proportional relationship between lifetime and yield of the two components was observed). Similar results to those shown in figs 1 and 3 have also been found for *C. reinhardtii* (not shown) and representative lifetimes and yields for *C. reinhardtii* at F_0 and F_{max} are given in table 1.

Preliminary studies have indicated that the constant, fast component, which does not noticeably change in yield or lifetime in response to the closure of PS II centres, has an enhanced yield (4 to 10–12%) at longer emission wavelengths (>710 nm) compared with 685 nm, is absent in BBY-type PS II oxygen-evolving preparations [18] at F_{max} and is modified in a *Chlamydomonas* mutant (F15) lacking PS I reaction centre proteins (not shown).

4. DISCUSSION

The deconvolution of the chlorophyll fluorescence decay kinetics shown above into at least 3 exponential components to fit the overall decay is in agreement with earlier works [1,2,4,6,7,11]. At the F_{max} level both the average lifetime and the parameters from our deconvolution are similar to those reported for green algae [1,2]. However, this is not the case at the F_0 level, where an average lifetime of 330 ps was found which is shorter than previously reported for *Chlorella* [1,2]. This difference could be explained by the different experimental apparatus and procedures used which allowed a lower F_0 level to be reached in this work. In fact, the low fluorescence state attained with *Chlorella* in [1] might reflect a state in which a small fraction of PS II is closed. If this is assumed then the component lifetimes reported in [1] fit well with the data shown in fig.3a for the same average lifetime. The data in [2] gave values slightly different from those in the earlier work [1] and those in figs 1a and 3a. At an F_0 corresponding to 470 ps the 3 exponentials were deconvoluted into a fast (130 ps), a middle (500 ps) and a slow (1.4 ns) component. Similar lifetime components and average lifetimes, at F_0, have been reported however, using barley chloroplasts suspended in the presence of high salt. Such differences are difficult to explain in terms of a species dependence but perhaps reflect the different experimental apparatus and procedures used. Furthermore, comparisons between data of different groups could be hindered due to the same, or different species, being grown under different growth conditions or perhaps being used at different stages of development (see [5]).

Here, the magnitude of the change in both the lifetime of the middle and slow components, on going from F_0 to F_{max} by photochemically closing the PS II reaction centres (fig.3a), is different from the data for green algae [1,2], and for chloroplasts of spinach [1,4], pea [1] and barley

[6,7]. It can be seen that there is an almost 5-fold increase in the lifetimes of both these components as Q_A is reduced; such large changes have not been observed previously (usually the change is only 2-fold). The relationship between the state of the PS II reaction centres and the fluorescence yield of the two slower components is less clear. The most consistent changes (fig.3b) suggest that as Q_A is reduced the middle component might be changed in some way to give rise to the slow component.

The finding of the 3 lifetime components and their behaviour as PS II reaction centres are closed have been used as the basis for many different interpretations concerning the organisation of the photosynthetic apparatus. Therefore, the novel results shown in fig.3 have led us to interpret the organisation of PS II somewhat differently from the previous works as discussed later. Until now the association of each component with a subset of PS II divided using the tripartite model of Butler [19] into peripheral LHC, PS II core antenna and PS II reaction centres has been carried out (see section 1). However, it has been shown that the middle component is almost unaltered in a mutant lacking LHC [6] and that a slow component is still present in a mutant lacking PS II [7]. Such observations cannot be accommodated by the above-mentioned associations and suggest that the components may have multiple origins. The presence of a long-lived component in the absence of PS II reaction centre proteins suggests that the charge recombination mechanism is not essential. Furthermore, mutant and developmental studies infer that the fast component originates from PS I and PS II while the middle component cannot arise solely from LHC [5–7].

Another idea put forward to explain the origin of the 3 lifetime components introduced the concept of PS II_α and PS II_β centres [9]. However, based on the reported properties and locations for such centres in which PS II_α units are connected and found in the granal membranes and PS II_β are isolated centres situated only in the stromal lamellae [20,21], this hypothesis cannot explain the above results. Both the 5-fold increase in the middle and slow component lifetimes (fig.3a) and their location in appressed, granal membranes would not be expected if these components represented PS II_β and PS II_α centres, respectively, firstly, because PS II_β is absent from BBY preparations [21] and, secondly, as isolated centres they would not exhibit a variable lifetime component but produce constant lifetime components which would change only in amplitude between the open and closed states.

The fluorescence from PS I-associated pigments has recently been confirmed to be a part of the fast component. On the basis of spectral distribution a 104 ps component has been ascribed to PS I [13] while other workers have resolved the fast decay into two components of 80 ps (PS I) and 180 ps (PS II). We have also found a 3-fold increase in yield of the fast component at wavelengths >710 nm compared with the emission at 685 nm. Furthermore, preliminary studies have shown that a fast (<100 ps) component is absent, at F_{max}, in BBYs and is greatly modified in a *Chlamydomonas* mutant lacking PS I.

Both the proportional increase in lifetimes of the middle and slow components with average lifetime (fig.3a), and fig.2, favour a model in which PS II reaction centres are well-connected, enabling energy transfer between reaction centres [16,22]. The large variable change in the slow component lifetime between F_0 and F_{max} suggests that this component cannot arise directly from charge recombination. If such a mechanism is involved then the recombination time must be rapid (<100 ps) otherwise extrapolation of the slow decay to zero yield in fig.3a would not be close to the origin. The fast recombination must then lead to the re-excitation of the antenna pigments allowing energy transfer between different PS II reaction centres to take place.

Because the lifetimes of the two variable components increase by the same degree and in parallel with the yield, upon closing the PS II reaction centres, the different fluorescing units producing these emissions must be connected to similar degrees and be associated with and dependent upon the PS II reaction centre [17]. They are also both situated in the granal lamellae as shown by the analysis of BBY-type preparations. However, a model of two sets of well-connected units infers that for each emission (middle and slow) the lifetime and yield should increase in a constant ratio during the fluorescence induction rise. The data of fig.3 show that such a relationship only holds for the first half of the average lifetime increase. For the second half, the yield of the slow component exhibits a

larger change than expected at the expense of that of the middle component. This trend could arise from uncertainties in the deconvolution but an analysis using simulated tri-exponential decay curves, in the presence of Gaussian noise, suggests that this is not the case. Other possible explanations might be that it arises because of either the proposed multiple origins of the decay components (see e.g. [7]) or the units giving rise to the middle component are being 'converted' into those centres producing the slow component. However, a 4-component deconvolution with slightly better fits to the experimental data than with 3 components leads to a situation where 2 components exhibit a proportional increase in both yield and lifetime (approx. 6-fold) as PS II traps are closed while the other 2 components remain constant in yield and lifetime (60 ps and 250 ps). This analysis therefore does not require any conversion of different fluorescing units and confirms the multiple origin hypothesis. Further work is now being carried out to try to verify and extend the conclusions presented above.

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