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Time-resolved chlorophyll fluorescence studies of photosynthetic membranes: resolution and characterisation of four kinetic components

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Room temperature single-photon timing measurements on intact green algae and higher plant thylakoids at low excitation energies have been analysed using a four-exponential kinetic model. Closing the Photosystem II (PS II) reaction centres produced two variable lifetime components (0.25–1.4 ns and 0.45–2.6 ns) and two constant components (50 and 220 ps) between the initial (F_0) and maximal (F_M) fluorescence levels. The yield of the variable components paralleled the changes in their lifetimes, while the yields of the constant emissions remained insensitive to the state of PS II. These observations indicate the presence of well-connected PS II centres favouring energy transfer between each other. Time-resolved emission spectra, at F_0 and F_M, show that the three 'slow' components have fluorescence maxima at 685 nm, while the fast decay has a maximum emission between 695 and 705 nm. Kinetic and spectral analyses of decays from a *Chlamydomonas* mutant and PS II-enriched membranes lacking Photosystem I (PS I) showed the absence of the fast, red-shifted component and only required a triexponential model to fit the decays. At F_M the three components retrieved from the PS II-enriched thylakoids were identical to the PS II-associated emissions of intact thylakoids. Such observations indicate the requirement for at least four kinetic components to describe the fluorescence decays of intact light-harvesting Chl a/b protein complex (LHC) containing photosynthetic material best. Furthermore, these data cannot be interpreted in terms of the α/β heterogeneity of PS II.

Introduction

The primary processes of photosynthesis are characterised by the highly efficient absorption of light energy by a complex light-harvesting pigment-containing system, followed by its rapid transfer to the photosynthetic reaction centres. The structural organisation of the pigment-protein complexes, within higher plants and green algae, is very heterogeneous, consisting of two types of photosystem, each being associated with its own pigment-containing polypeptides [1]. The situation is further complicated by the presence of various PS II heterogeneities: α and β centres [2], Q_1 and Q_2 [3], B and non-B type [4] and Q_H and Q_L [5]. Therefore much uncertainty still exists concerning the detailed structural organisation and energy transfer pathways of the photosynthetic apparatus. One method by which these problems are currently being investigated is the analysis of chlorophyll fluorescence-decay kinetics using a combination of single-photon counting and low-intensity

Abbreviations: PS I, Photosystem I; PS II, Photosystem II; Chl, chlorophyll; LHC, light-harvesting Chl a/b protein complex; Ph, pheophytin; F_0, initial fluorescence level; F_M, maximum fluorescence level; F_V, variable fluorescence ($F_M - F_0$); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Mes, 4-morpholineethanesulphonic acid.

picosecond laser excitation [6–10].

Observations have suggested that the sum of at least three exponential components are necessary to describe the in vivo fluorescence decays of LHC-containing photosynthetic material [11–15]. For a mechanistic description of the decay, in terms of structure and transfer, it is necessary to assign each component to functional constituents of the photosynthetic system. The fast decay [80–150 ps] was originally attributed to the core antenna chlorophylls of open PS II centres [11,13,15]. The middle component (300–700 ps) was ascribed to LHC [11,13,15] or to PS II in a different redox state [14]. The long-lived decay (1.2–2.2 ns) was associated with antenna reexcitation following charge recombination of P-680$^+$ and reduced pheophytin when PS II was closed [10,15], and was regarded to be exclusively responsible for variable fluorescence (F_V). The opposite dependence of the fast and slow component yields and the small difference in component lifetimes favoured the organisation of PS II as separate units with only a very limited degree of energy transfer [13]. Such observations and ideas have recently been questioned due to a 4–5-fold increase in slow component lifetime on closing PS II [8]. Studies using mutants lacking LHC [16] or PS II [17] also suggest that the reported origins of the decays may be incorrect.

An alternative hypothesis for the origins of the three components was proposed by Butler et al. [18] by introducing α- and β-type PS II centres and a PS I emission. It was proposed that the fast decay was a composite of PS I and open PS II$_\alpha$ centre emissions, the middle lifetime represented PS II$_\beta$ centres and the slow component arose from closed PS II$_\alpha$ centres [19]. Other groups have since attempted to relate their experimental findings to a similar model. Karukstis and Sauer [10] have, however, suggested that it is not possible to distinguish individual α and β centre components. Recently, Holzwarth et al., as a result of time-resolved emission and excitation spectral analyses, have suggested that at least four kinetic components are required to fit the overall decay [9]. They have divided the original fast decay into PS I (80 ps) and open PS II$_\alpha$ centre (180 ps) emissions, with the old 'middle' component originating from open (500 ps) and closed (1.2 ns) PS II$_\beta$ centres and the long-lived decay (2.2 ns) arising from closed PS II$_\alpha$ centres. The additional complexity of the fluorescence decay signal has also been reported by Gulotty et al. [20] who have proposed, following mutant studies, that the three-component model is insufficient. We have previously mentioned that a four-component model may produce a better fit to the observed decays [8].

In this present work is shown the effect of PS II trap closure on the lifetimes and yields of components derived from a free-running four-exponential model. The origin of each component is characterised by time-resolved emission spectral analyses at F_0 and F_M as well as the analysis of PS II-enriched thylakoid membranes and a *Chlamydomonas* mutant, both lacking PS I.

Materials and Methods

Chlorella pyrenoidosa and *Chlamydomonas reinhardtii* were grown as previously described [8]. PS II-enriched oxygen-evolving thylakoid membranes were prepared either as in Ref. 21 for 'BBY's' (particles called after Berthold, Babcock and Yocum) or as in Ref. 22 for Murata particles. Broken chloroplasts were prepared as previously described [23]. For experimentation, the algae were diluted to 20 μg Chl/ml in growth medium, the 'PS II preparations' were diluted to give final concentrations of 20 μg Chl/ml, 0.3 M sucrose, 2.5 mM Mes (pH 6.5), 10 mM KCl, 5 mM $MgCl_2$, and the chloroplasts were diluted to give final concentrations of 20 μg Chl/ml, 0.4 mM sorbitol, 0.01 M Tricine (pH 7.8), 10 mM KCl, 5 mM $MgCl_2$.

All fluorescence measurements were carried out and analysed using the criteria and apparatus as previously described [8]. The single-photon counting apparatus and deconvolution programme were checked by measuring and analysing the fluorescence decay of oxazine in methanol (Fig. 1). It can be seen that only a single-exponential decay with a lifetime of 785 ps was required to fit the data, thereby showing that the system functioned correctly.

Time-resolved emission spectra were calculated as follows: for each wavelength investigated a fluorescence decay was measured and deconvoluted using either three or four free-running com-

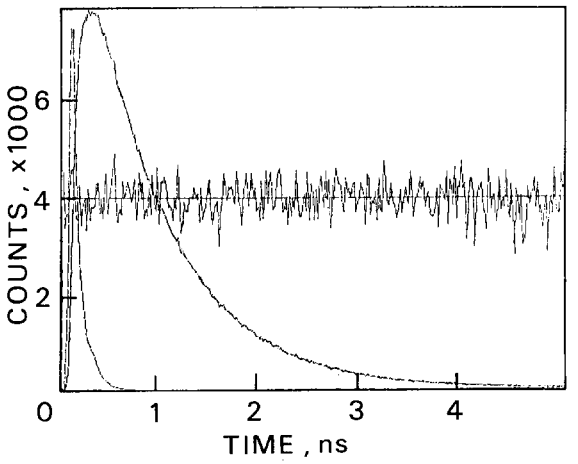

Fig. 1. Excitation profile and fluorescence decay of oxazine in methanol measured at 685 nm, fitted by one exponential giving a lifetime of 785 ps with a χ^2 of 0.92. The weighted differences between the measured and fitted curves are shown in the centre of the plots (scale +10 to −10).

ponents. The calculated lifetimes for each component at each wavelength were averaged to give a mean lifetime for each component. Each decay was reanalysed by linear regression using the mean lifetime of each component to generate the component amplitudes at each wavelength. This procedure introduced only small increases in the reduced χ^2 when compared to the free-running deconvolution.

Simulated decay curves were generated by using two mathematical functions to define both the fluorescence (F) and the excitation function (G). These were chosen to verify the relation $F = G \times S$, where S = the sum of three or four exponential components with parameters analogous to those found experimentally and G = the difference of two exponentials. The width of G was chosen to be in the same time range as the true experimental function. This method avoids the use of the numerical convolution algorithm used in the deconvolution programme to produce the simulated decays, and provides an absolute check of both the numerical convolution algorithm and the minimisation algorithm.

Results

It was found in Ref. 8 that the changes in lifetime and yield of each component, brought about by PS II trap closure, could not be easily interpreted using the triexponential model. From the time-resolved emission spectral analyses, the predicted constant lifetime relationship with emission wavelength was not observed when using a three-component model (Fig. 2A), inferring that such an analysis was in fact inadequate. The decays were therefore deconvoluted using four free-running lifetime components which led to the removal of any wavelength dependence (Fig. 2B).

The ability to deconvolute four lifetime components was tested using simulated decay curves generated in the presence of gaussian noise (see Fig. 3). Table I shows the theoretical and deconvoluted lifetime and preexponential factor (A_{exp})

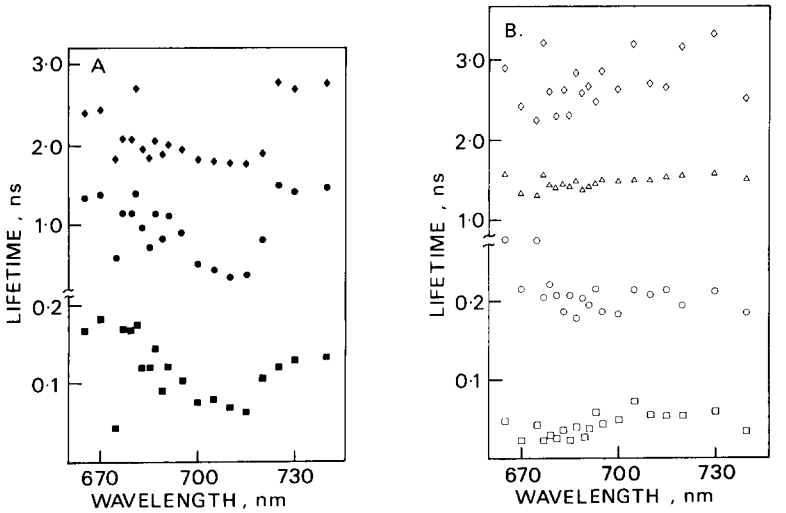

Fig. 2. The effect of emission wavelength on the deconvoluted lifetimes of the kinetic components from *Chlorella* using (A) a triexponential and (B) a quadriexponential model at F_M.

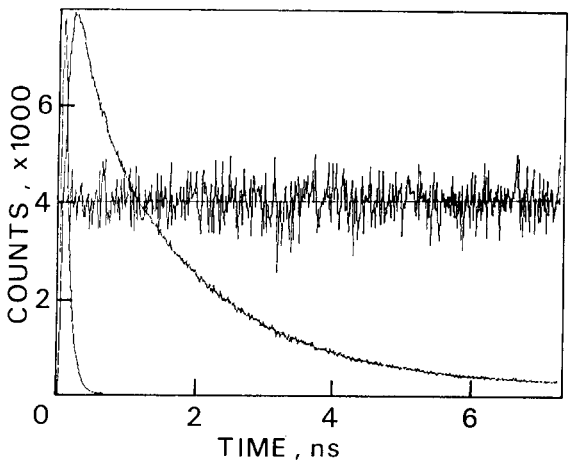

Fig. 3. Simulated excitation profile and four-component decay in the presence of noise and fitted by four free-running kinetic components (see Table I). The weighted differences between the synthetic and fitted curves are shown in the centre of the plots (scale + 10 to − 10).

values for a series of simulated curves representing different fluorescence levels between F_0 and F_M. It can be seen that the parameters were recovered from the curves with a reasonable degree of accuracy, except at F_0. When the four component curves were analysed using only three exponentials, good χ^2 values were still retrieved, and a trend between variable component lifetimes and yields similar to that previously observed [8] for PS II trap closure was produced (not shown). However, three-component simulations could not be modelled using a four-component analysis as this led to the production of negative amplitude components (not shown). We are therefore confident of the values obtained from our free-running quadri-exponential analyses carried out on experimental data, except at close to F_0.

Fig. 4A shows the evolution of the lifetime of

TABLE I

DECAY COMPONENTS OF SIMULATED FLUORESCENCE DECAY CURVES DECONVOLUTED USING A THREE (3)- AND FOUR (4)-EXPONENTIAL KINETIC MODEL

		Lifetime (ns)				χ^2
Theoretical	τ	0.500	0.290	0.230	0.040	
	A_{exp}	0.24	0.31	0.12	0.33	–
3	τ	0.511	0.290	–	0.039	0.903
	A_{exp}	0.21	0.42		0.37	
4	τ	0.520	0.302	0.135	0.035	0.912
	A_{exp}	0.19	0.41	0.03	0.37	
Theoretical	τ	1.200	0.690	0.230	0.040	–
	A_{exp}	0.24	0.31	0.12	0.33	
3	τ	1.129	0.541	–	0.054	1.08
	A_{exp}	0.34	0.26		0.36	
4	τ	1.250	0.773	0.300	0.038	1.07
	A_{exp}	0.18	0.32	0.13	0.37	
Theoretical	τ	2.500	1.450	0.230	0.040	–
	A_{exp}	0.24	0.31	0.12	0.33	
3	τ	2.149	0.860	–	0.070	1.08
	A_{exp}	0.47	0.17		0.36	
4	τ	2.548	1.508	0.245	0.042	1.05
	A_{exp}	0.20	0.31	0.09	0.35	
Theoretical *	τ	3.000	1.500	0.200	0.05	–
	A_{exp}	0.10	0.40	0.20	0.30	
3	τ	2.235	1.082	–	0.098	1.13
	A_{exp}	0.32	0.26		0.42	
4 *	τ	3.001	1.520	0.208	0.041	1.05
	A_{exp}	0.09	0.39	0.18	0.35	

* These data are represented in Fig. 3.

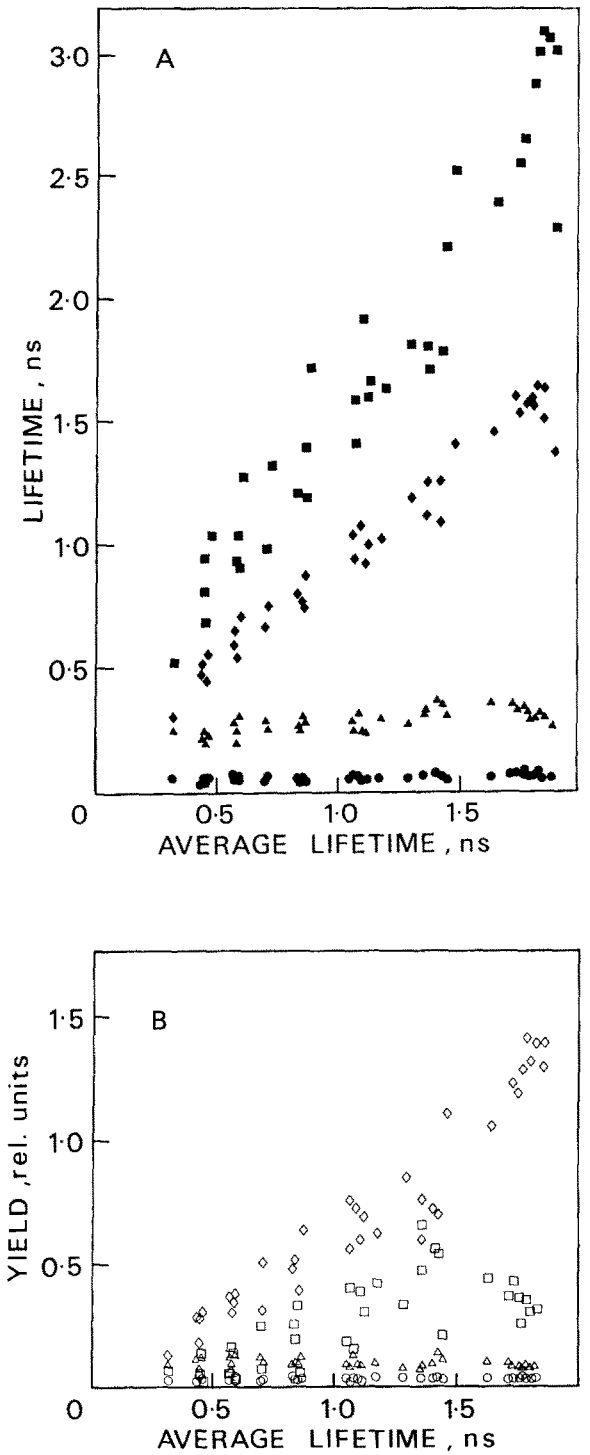

Fig. 4. The effect of PS II trap closure, as measured by the average fluorescence lifetime, on (A) the lifetime and (B) the yield of the four kinetic components of the fluorescence decay of *Ca. pyrenoidosa*.

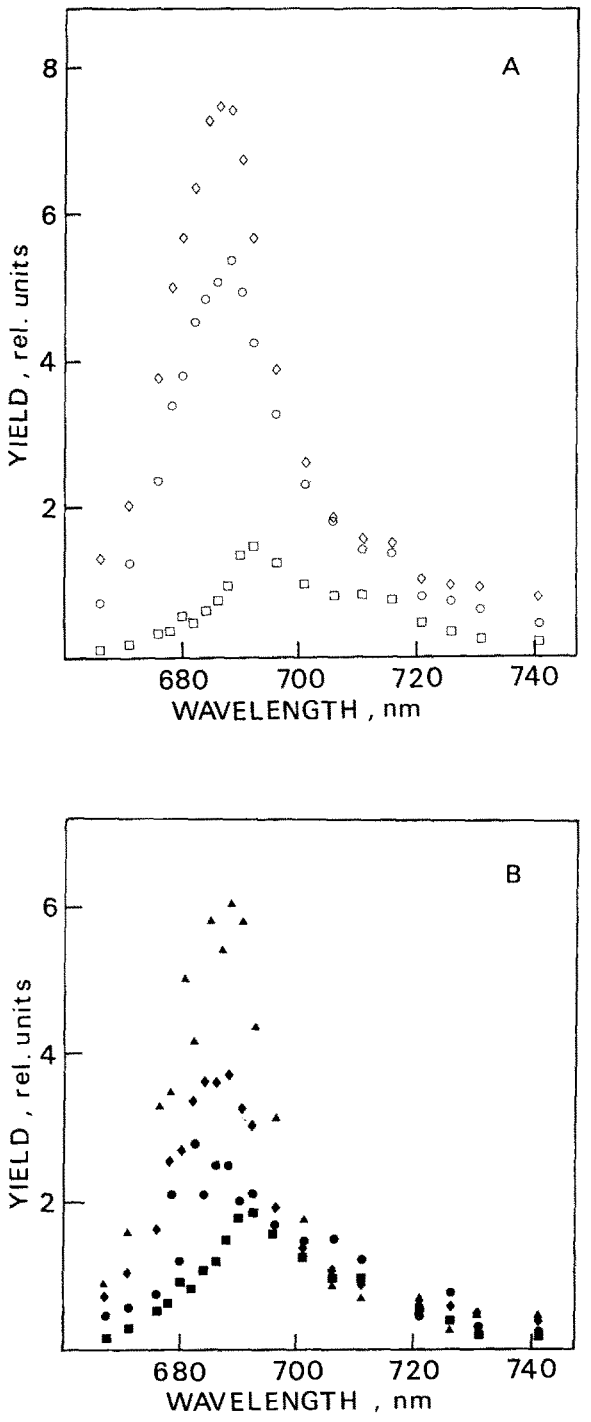

Fig. 5. The time-resolved emission spectra of the individual decay components of *Ca. pyrenoidosa* at the F_0 level using (A) a free-running triexponential model (◇, 0.436 ns; ○, 0.192 ns; □, 0.043 ns), and (B) the quadriexponential model (◆, 0.525 ns; ▲, 0.29 ns; ●, 0.22 ns; ■, 0.055 ns), with fixed lifetime values predicted from Fig. 4.

TABLE II

DECAY COMPONENTS OF THE CHLOROPHYLL FLUORESCENCE FROM INTACT *CA. PYRENOIDOSA* AND *C. REINHARDTII* CELLS AND BROKEN CHLOROPLASTS OF SPINACH MEASURED AT F_0 AND F_M

A, relative amplitude of each component.

			Lifetime (ns)				τ_{mean}
Ca. pyrenoidosa	F_0	τ	–	0.447	0.223	0.059	0.315
		A (%)		44	44	12	
	F_M	τ	2.589	1.444	0.224	0.057	1.785
		A (%)	20	74	4	2	
C. reinhardtii	F_0	τ	–	0.609	0.190	0.039	0.432
		A (%)		51	30	9	
	F_M	τ	2.449	1.417	0.231	0.033	1.948
		A (%)	57	38	3	2	
Spinach chloroplasts	F_0	τ	–	0.581	0.193	0.043	0.290
		A (%)		28	59	13	
	F_M	τ	2.778	1.432	0.250	0.044	1.955
		A (%)	44	50	5	1	

each component as PS II reaction centres are closed, as indicated by the average lifetime, following a free-running four-component deconvolution of the experimental fluorescence decays, except at F_0. The striking observations are firstly the lack of a long-lived component at F_0, as previously reported [8], and secondly the proportional increase in the lifetime of two components on increasing the number of closed PS II centres. The lifetimes of the two remaining components remained relatively insensitive to PS II photochemistry, with approximate lifetimes of 50 ps and 220 ps. These measurements have been repeated many times using intact *Chlorella pyrenoidosa* and *Chlamydomonas reinhardtii* cells and isolated broken chloroplasts from spinach, lettuce and pea and have always yielded changes in component lifetimes that are similar to those in Fig. 4A (see Table II).

Fig. 4B shows the variation in component yield as the average lifetime is altered. As for the corresponding lifetime measurements (Fig. 4A), there are two variable and two constant yields when PS II centres are closed. Although a large variation is seen in the yields probably arising from the large number of mathematical solutions to describe the data using a four-component analysis, it appears that the observed lifetime changes are mirrored by similar modifications in yield.

Time-resolved emission spectra were carried out at F_0 and F_M. At F_0 it was adequate to represent the total decay by a 'simple' triexponential model. The emission spectra at F_0 are shown in Fig. 5A

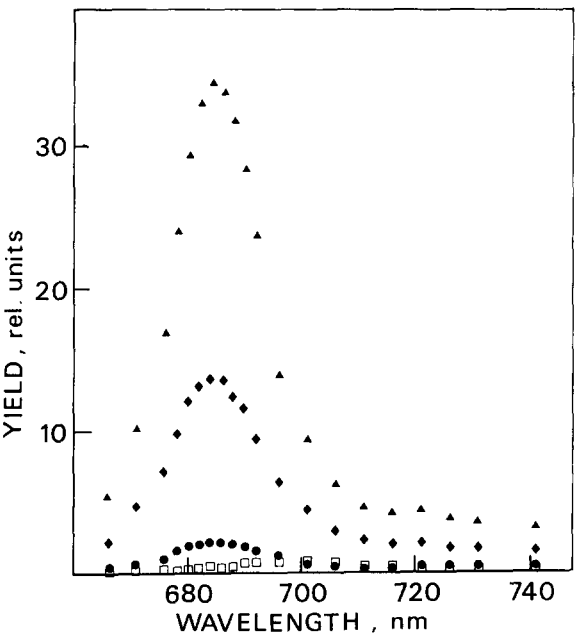

Fig. 6. The time-resolved emission spectra of the individual decay components of *Ca. pyrenoidosa* at the F_M level (□, 0.057 ns; ●, 0.224 ns; ▲, 1.444 ns; ◆, 2.589 ns).

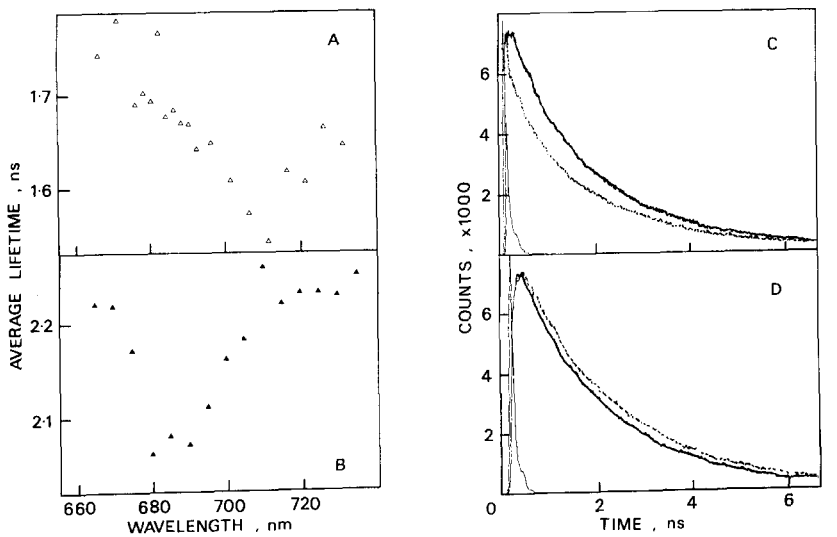

Fig. 7. The wavelength dependence of the average lifetime for (A) wild type and (B) PS I-lacking mutant cells of *C. reinhardtii* at F_M. The excitation profile and undeconvoluted fluorescence decays measured at 685 nm (————) and 710 nm (— — —) for (C) wild type and (D) PS I-lacking mutant cells of *C. reinhardtii* at F_M.

where it can be seen that two components with lifetimes of 190 ps and 450 ps have emission maxima at 685 nm while a fast decay (60 ps) exhibits a broad spectrum with a peak at 690–695 nm and a large shoulder at 705–715 nm. The form of the fast emission suggests that it is composed of at least two components. A four-component analysis was carried out using lifetime values predicted from Fig. 4A. This approach produced the emission spectra of Fig. 5B which shows three components peaking at approx. 685 nm and a fast decay with a red-shifted maximum at 695 nm. Such an observation is similar to that previously reported [9] for a situation close to F_0 where the decay was analysed with two fixed fast lifetime components of 80 ps and 180 ps. From the component yields produced using this procedure, it can be seen that 60–70% of the F_0 fluorescence is homogeneous with F_V fluorescence.

Fig. 6 shows the time-resolved emission spectra at F_M using the four-component analysis. It can be seen, that the three 'slow' components have emission maxima at 685 nm, while the fastest component (57 ps) exhibits a red-shifted spectrum with a maximum yield at 700 nm.

The increase in the relative weight of the fast decay, compared with the other components, can easily be observed from either a plot of the aver-

TABLE III

DECAY COMPONENTS OF THE CHLOROPHYLL FLUORESCENCE DECAYS FROM 'BBY' AND 'MURATA' PARTICLES MEASURED AT F_0 AND F_M

A, relative amplitude of each component

			Lifetime (ns)			τ_{mean}
BBY	F_0	τ	0.385	0.178	0.042	0.233
		A (%)	33	59	8	
	F_M	τ	2.491	1.233	0.235	1.530
		A (%)	27	68	5	
Murata	F_0	τ	0.405	0.184	0.041	0.240
		A (%)	29	62.5	8.5	
	F_M	τ	2.366	1.347	0.244	1.578
		A (%)	30	65	5	

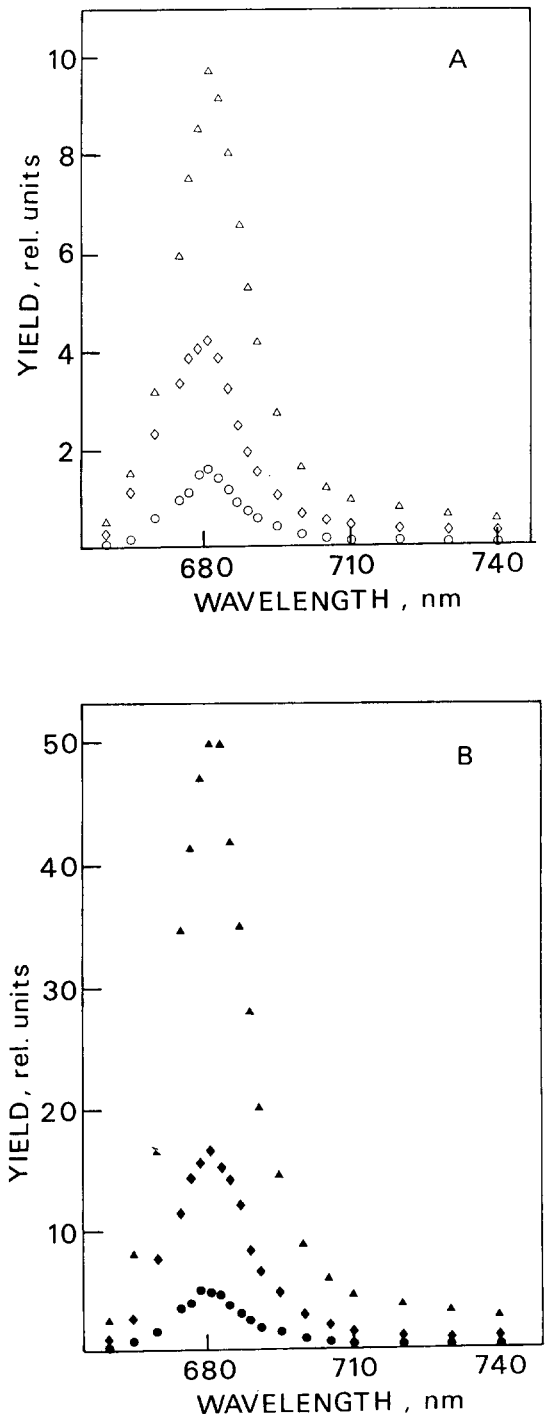

Fig. 8. The time-resolved emission spectra of the individual decay components of 'Murata' particles at (A) F_0 and (B) F_M. The lifetimes of each component are: △, 0.209 ns; ◇, 0.420 ns; 0.062 ns at F_0, and ▲, 1.211 ns; ◆, 2.255 ns; ●, 0.315 at F_M.

age lifetime versus emission wavelength (Fig. 7A) or by comparing nondeconvoluted decay curves measured at 685 nm and 710 nm (Fig. 7C). Similar observations are not found when using a *Chlamydomonas* mutant lacking PS I reaction centres. It can be seen from Fig. 7B that the average lifetime of this mutant produces a minimum at 685 nm while the decay at 685 nm appears to be slightly faster than that measured at 710 nm (Fig. 7D). This mutant does not exhibit a fast, red-shifted lifetime component (not shown). Such observations suggest that this component originates from PS II-associated pigments.

This conclusion is strengthened from the analysis of PS II-enriched thylakoids which also lack PS I and which do not produce a fast, red-shifted fluorescence component. The decays produced by either 'BBY's' or 'Murata' particles only require a three-component model to described the overall decays. This is concluded from the wavelength independence of the three components (not shown), as well as from good χ^2 values. Each kinetic component exhibits similar emission characteristics at both F_0 (Fig. 8A) and F_M (Fig. 8B), which follow the total fluorescence yield spectrum. Table III shows the lifetime and amplitude values for both types of PS II-enriched preparations at F_0 and F_M. At the maximum fluorescence level, the components are similar to the PS II-associated emissions from intact thylakoid membranes (compare Table II with Table III). However, at F_0 the situation appears to be complicated by the presence of a fast PS II component (42 ps) which is not seen at F_M.

Discussion

Several years ago it was proposed that the room temperature chlorophyll fluorescence decay emission, as measured using single-photon counting and picosecond excitation techniques, had multiexponential characteristics (Refs. 11, 13, 15). The deconvolution of such fluorescence signals into at least four kinetic components, as reported above, is in close agreement with recent observations made by Holzwarth et al. [9]. Although such a complex mathematical analysis is open to certain criticisms, we feel that there is good reason to believe that the three-component model is not

adequate to describe the overall fluorescence decay. This can be seen from the extreme wavelength dependence of the calculated lifetimes using a triexponential analysis (Fig. 2A) and its subsequent removal when using a four-component model (Fig. 2B). The emission spectra measured at F_0 and analysed by a three-component model (Fig. 5A) suggest that the rapid decay is composite in nature, consisting of a super position of at least two spectrally distinct emissions. Although an analysis of the decay at F_0 using four free-running lifetime components is not easily reproducible, when deconvoluted using fixed lifetime values, extrapolated from Fig. 4A, removal of the composite nature was achieved (Fig. 5B). Furthermore, a non-linear relationship between the change in lifetime and yield of the so-called 'middle' and 'slow' lifetime components on closing PS II reaction centres was found in Ref. 8 using a three-component analysis. The quadriexponential kinetic model, described in this work, generates changes in component yield and lifetime which follow the predicted $\phi \sim \tau$ relationship.

The change in the lifetime of the four components when PS II centres are closed shows the presence of two variable and two constant lifetime components between F_0 and F_M. The variable decays which make up the major contribution to the F_V produce similar increases in lifetime and yield (approx. 5–6-fold) as seen from Table II and Fig. 4. The two minor components have lifetimes and yields which are relatively insensitive to the state of the PS II reaction centres. The proportional increase in lifetime and yield of two variable components clearly favours a model in which PS II reaction centres are organised so as to facilitate good excitation energy transfer between centres (i.e., well connected, see Refs. 24 and 25). This conclusion disagrees with many of the earlier interpretations (e.g., Refs. 10 and 15) and with a more recent report [9] of lifetime data in which the opposite dependence of the yield of two components with relatively constant lifetimes was seen. This trend is expected for a model in which there is no energy transfer between PS II centres (i.e., an isolated model). Furthermore, as a result of the parallel increase in both yield and lifetime of the two variable decays, the recombination luminescence theory for variable fluorescence [see Ref. 26] can not be evoked unless the reinjection time of excitons into the antenna after charge recombination of P-680$^+$ and Ph$^-$ is very short (less than 100 ps). Therefore, the proposed origin of the slow lifetime component appears to be incorrect.

The emission spectra presented in Figs. 5 and 6 (for F_0 and F_M, respectively) show that at both fluorescence levels there are three decay components with emission maxima at 685 nm, probably attributable to PS II-associated pigments, and a rapid component which gives rise to a far-red shifted emission (λ_{max}, 695–705 nm), perhaps representing a room temperature PS I emission [see also Refs. 8, 9, 20 and 27]. The increase in relative amplitude of the fast component, with respect to the other components at longer wavelengths can be easily seen from Fig. 7A and C. The relative proportion of the fast decay, compared with the other emissions, shows a maximum between 705 and 710 nm, as seen from the position of the minimum in Fig. 7A, which is in agreement with a previous steady-state fluorescence report concerning room temperature PS I emission [28]. The PS I origin of the fast (50 ps) component is confirmed by the analyses of fluorescence decays from a *Chlamydomonas* mutant lacking PS I (Fig. 6B and D) in which no red-shifted fast component is observed. Consistent with this proposal is the absence of this component in PS II-enriched thylakoid peparations as seen from the emission spectra at F_0 (Fig. 8A) and F_M (Fig. 8B), in which all three components necessary to model the decays show maximum emissions at 681 nm.

The three emissions obtained from the analysis of PS II-enriched thylakoid membranes produced lifetime values at F_M which were similar to the PS II-associated components exhibited by intact thylakoids (compare Tables II and III). However, at F_0 a low-yielding PS II emission with a fast lifetime (40 ps) was seen which was not found at F_M. This observation, along with the data of Fig. 6B and D for the PS I mutant, might indicate the presence of a fast PS II-associated component. However, the description of the PS II preparations by a triexponential decay model, in the absence of PS I, confirms the requirement for at least four kinetic components to describe the decays of intact thylakoids.

We therefore conclude that the rapid, constant

decay is a PS I emission, insensitive to PS II trap closure, as previously suggested [8,9,18–20,27] although at F_0 it may also have a PS II contribution. The remaining three components are all associated with PS II. The two variable lifetime emissions can not be interpreted by the α/β PS II heterogeneity [2,29] because they do not fit certain previously reported characteristics for such centres. They both appear in PS II-enriched membranes which have been reported to contain only PS II_α [30]. Both emissions exhibit 5–6-fold increases in both yield and lifetime when PS II traps are closed, and are therefore not characteristic of isolated PS II_β centres. They both have similar fluorescence emission spectra, which is not in accord with the proposition that β centres show maximum emission at 730 nm [31] and maximum absorption at 690 nm [32]. We propose that the slowest, variable emission might represent fluorescence from reexcited LHC, while the faster, variable component could arise from reexcited Chla *a* antenna molecules close to PS II. The constant (220 ps) component might originate from excitons lost before reaching the reaction centres, perhaps arising from loosely coupled LHC which might represent the proposed 'mobile pool' of LHC believed to bring about the redistribution of energy in response to LHC-phosphorylation [33]. It is interesting to note that at F_0 the 180 ps emission, perhaps equivalent to our 220 ps component, was preferentially excited by 652 nm light as opposed to 622 nm [9].

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