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Modification of room-temperature picosecond chlorophyll fluorescence kinetics in Photosystem-II-enriched particles by photochemistry

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Room-temperature single photon timing measurements on Photosystem-II (PS II-) enriched thylakoid fragments at low excitation energies indicate the presence of three kinetic decay components of chlorophyll fluorescence arising from PS-II-associated pigments. Closing the PS II reaction centres produced three variable components, with lifetime values of 0.02–0.25, 0.15–0.90 and 0.35–2.0 ns, between the initial (F_0) and maximal (F_m) fluorescence levels. The yield of each component paralleled the changes in their respective lifetimes, indicating the presence of well-connected PS II reaction centres favouring energy transfer between each other. These changes show that variable chlorophyll fluorescence (F_v) does not arise from one specific origin. The extent of the modifications and the observed relationship between component lifetime and yield, on closing PS II reaction centres, cannot be explained by either the delayed fluorescence (charge recombination) hypothesis of Klimov and co-workers (Klimov, V.V. et al. (1978) Dokl. Akad. Nauk. SSSR 242, 1204–1207) or the proposed changes and origins put forward by Holzwarth and co-workers (Holzwarth, A.R. (1986) Photochem. Photobiol. 43, 707–725; Holzwarth, A.R. et al. (1985) Biochim. Biophys. Acta 807, 155–167).

Introduction

Studies concerning the lifetime of chlorophyll fluorescence after picosecond excitation of photosynthetic organisms containing light-harvesting Chl *a*/*b* protein complexes (LHC) have revealed multiexponential decay kinetics (see reviews, Refs. 1, 2). The overall decay can usually be statistically well defined by three components, which undergo complex changes in both fluorescence lifetime and yield on closing Photosystem II (PS II) reaction centres to photochemistry (e.g., Refs. 3–5). Recent studies have suggested, however, that this triexponential model is oversimplistic and that the overall decay contains at least four components [6–8], with lifetime values of approximately 50 ps, 250 ps, 0.25–1.4 ns and 0.45–2.6 ns, for *Chlorella pyrenoidosa* [8]. It has been shown that the two rapid components are relatively insensitive to PS II trap closure, while the two longer-lived decays give rise to variable fluorescence (F_v) [8].

The origins of each component are still not clear. It has been shown from time-resolved emission spectra [7–9] and from mutant studies [10] that part of the rapid decay arises from Photosystem I (PS I) as well as from PS II. It was originally

Abbreviations: Chl, chlorophyll; PS, Photosystem; LHC, light-harvesting Chl *a*/*b* protein complex; Q_A, primary stable electron acceptor of PS II; Mes, 4-morpholineethanesulphonic acid; F_m, maximum chlorophyll fluorescence; F_0, minimum chlorophyll fluorescence; F_v, variable chlorophyll fluorescence.

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proposed that the slow component could originate from PS II radical pair recombination between P-680 and reduced pheophytin, following the work of Klimov and co-workers (see Ref. 11). This idea, however, has not been well substantiated because of (a) the reported 4–5-fold increase in the lifetimes and yields of the two, slow fluorescence decay components [8] and (b) the fact that photosynthetic mutants lacking PS II reaction-centre proteins still give rise to similar long-lived components [10,12]. However, it has been shown, by subtracting the F_m decay from the F_0 decay, that there exists a 200 ps lag component in the so-called F_v decay which was believed to be indicative of the charge recombination process [13].

Current ideas concerning the origin of the different kinetic components are based on the hypothesis first put forward by Butler and colleagues [14,15] that the lifetime data could be interpreted in terms of the α/β-type of PS II heterogeneity (e.g., see Ref. 16). Holzwarth et al. [6], as a result of time-resolved emission and excitation spectral analyses carried out at F_0 and F_m, have proposed that a constant 80 ps component arises from PS I, a 180 ps decay is due to open PS II_α centres, the original 'middle' component comes from open (500 ps) and closed (1.2 ns) PS II_β while the long-lived decay (2.2 ns) is emitted by 'dead' chlorophyll, at F_0, and closed PS II_α centres at F_m. However, the concept of α and β centres in terms of PS II centres of different antenna size [19] located in different areas of the thylakoid membrane [20] has recently been questioned [21], and therefore such an interpretation carries a certain degree of uncertainty.

In this work a well-characterised PS-II-enriched preparation [22] has been used to simplify the photosynthetic system. This material still evolves oxygen at good rates and is very stable [22]. It contains only very small amounts of cytochrome *f*, cytochrome b_6 and PS I [22,23]. Preliminary observations concerning this type of PS-II-enriched membrane have shown that a red-shifted, rapid (40 ps) decay component is absent (or not detectable due to the presence of only very small concentrations) and that the overall decay is best described by three kinetic components, each having their maximum emissions at 681 nm at room temperature [8]. Here we show the changes in both yield and lifetime of each individual decay component as PS II reaction centres are progressively closed.

Materials and Methods

PS-II-enriched membranes were prepared according to Ref. 22. For experimentation the preparation was diluted to give final concentrations of 20 μg Chl/ml, 0.3 M sucrose, 2.5 mM Mes (pH 6.5), 10 mM KCl and 5 mM $MgCl_2$. All fluorescence measurements were carried out and analysed using the apparatus and criteria already described in Ref. 5. The F_0 was produced by a flow method (see Ref. 5) in the presence of 0.1 mM potassium ferricyanide. The F_m was generated under stationary conditions in the absence of DCMU. Intermediate fluorescence levels were produced by flowing the sample at different flow rates, with and without a saturating preillumination to reduce all Q_A, in the presence and absence of 0.1 mM potassium ferricyanide (see Ref. 5).

Results

Fig. 1 shows the instrumental response function to scattered light measured at 626 nm (FWHM = 60 ps) and the chlorophyll fluorescence decay of the PS-II-enriched membranes measured at 681 nm, following excitation at 626 nm, at F_0 (A) and F_m (B). Three exponential decay components were required to fit the overall decay at both the minimum and maximum fluorescence levels (see Ref. 8). The best fit of a triexponential model decay is superimposed with the experimental decay. The weighted difference between the experimental decays and the fitted decays is shown in the centre of Fig. 1. When Q_A was oxidised in the presence of potassium ferricyanide (Fig. 1A), the three individual decay components had short-lived lifetimes in the order of 20, 140 and 300 ps, while when Q_A was reduced (Fig. 1B) the lifetimes retrieved were much longer ($\tau = 0.25$, 0.9 and 2.0 ns). Several intermediate levels of fluorescence were measured, corresponding to different degrees of Q_A oxidation/reduction, and the overall decays were always best fitted by three exponential components (decays not shown). Two components introduced systematic deviations in the weighted

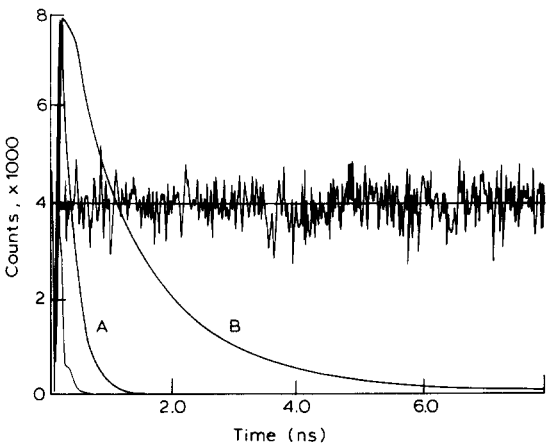

Fig. 1. Room-temperature chlorophyll fluorescence decays of PS-II-enriched particles measured at 681 nm at (A) F_0 (all Q_A oxidised in the presence of 0.1 mM potassium ferricyanide) and (B) F_m (Q_A reduced in the absence of DCMU). The excitation profile shown has a width of approx. 60 ps (FWHM). The best fit of a tri-exponential model is superimposed with the experimental decays. The weighted difference between the experimental decays and the fitted decays is shown in the centre of the plot (scale +10 to −10).

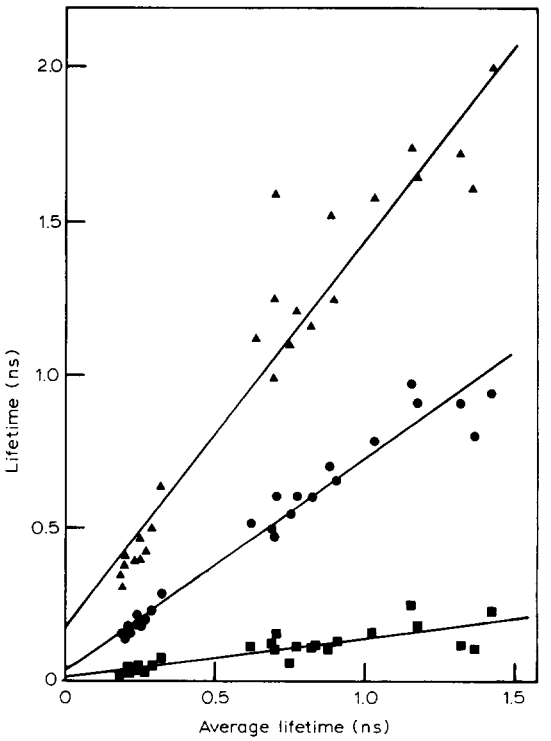

Fig. 2. The effect of PS II trap closure, as monitored by the average fluorescence lifetime, on the lifetimes of the three deconvoluted decay components exhibited by PS-II-enriched particles. The lines are the best fit through the datum points following correlation and least-squares regression analyses.

residuals and produced poorer chi-square values (our criteria for testing a good fit) while four decay components led to only a slight lowering (< 0.1) of the chi squares and produced an additional component with an insignificant amplitude.

Fig. 2 shows the evolution of the lifetimes of the three components as PS II reaction centres are closed (as indicated by the average lifetime). The average lifetime was used because, in this case, it is a linear indicator of the total fluorescence yield (data not shown; however, see Ref. 5). The proportional increase in each of the components lifetimes, by a factor of approx. 6, on going from F_0 to F_m was observed; the rapid decay increasing from 30 ps to 230 ps, the middle component going from 150 ps to 900 ps with the slow decay changing from 350 ps to 2 ns. It might be suggested that the 150 ps component gives rise to the 2 ns decay and that the 350 ps component becomes the 900 ps decay at F_m (as proposed for the 'intact' system in Refs. 2, 6). However, the results of a statistical analysis to find the correlation coefficient (r) for the different situations shows that $r = 0.975$ for the 'middle' component and $r = 0.922$ for the 'slow' component (as shown in Fig. 2) while the other possible situation leads to lower r values of 0.87 and 0.90, respectively. Fig. 3 shows the change in the relative fluorescence yield of each kinetic component in response to PS II trap closure, again depicted by the change in average lifetime. It can be seen that, as for the lifetimes of the three components, the yield of each of the individual decays exhibited similar increases which appear to parallel the changes in their respective lifetimes. Correlation analyses for the data in Fig. 3 confirm that it is the 150 ps decay which progressively gives rise to the 900 ps component and that the 350 ps decay gradually produces the 2 ns component at F_m ($r = 0.9$ and 0.98 for the proposed situation shown in Fig. 3 with $r = 0.84$ and 0.96 for the alternative possibility).

It has been seen that preferential excitation of Chl *b* with 652 nm light does not change the relative fluorescence amplitudes of the three decay

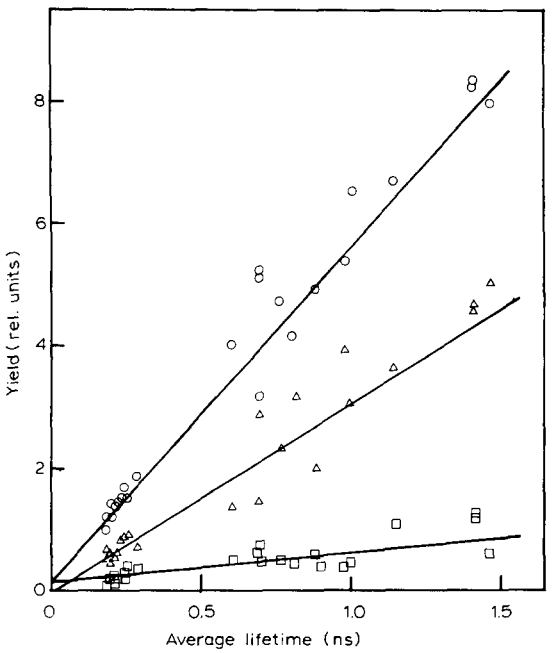

Fig. 3. The effect of PS II trap closure, as monitored by the average fluorescence lifetime, on the relative yields of the three deconvoluted kinetic components exhibited by PS-II-enriched particles. The lines are the best fit through the datum points following correlation and least-squares regression analyses.

components exhibited by these PS-II-enriched membanes, which is in disagreement with the observations made using the more complex in vivo situation as studied in Refs. 2 and 6.

Discussion

The lifetime of each component is determined by various deactivation and energy transfer rate constants. The continuous change in component lifetime (Fig. 2), the parallel increase in yield of each component (Fig. 3) and the insensitivity of the individual decays to preferential excitation of Chl *b* suggest that the fluorescing pigment-protein complexes are connected to each other and, in some way, to the PS II reaction centre. The extent of the observed changes suggest an extensive degree of connectivity between the different PS II antenna proteins favouring excitation energy transfer as already proposed [24,25]. The fact that each component is equally affected by PS II closure infers that they are each connected to the same degree. It seems improbable that there are three different types of well-connected antenna/reaction centre system located in granal thylakoid membranes and therefore the observed kinetic components could represent different antenna proteins connected to the same reaction-centre complex.

It has been proposed that the long-lived decay component arises from the charge recombination between oxidised P-680 and reduced pheophytin and therefore it was suggested that F_v was infact a delayed emission and not prompt fluorescence [11]. However, the large and proportional changes in lifetime and yield along with the variable nature of each PS II component, as PS II reaction centres are closed to photochemistry, can not be accommodated by a slow (ns) recombination mechanism. If charge recombination takes place during the time of our measurements, then it must be a rapid (less than 100 ps) process leading to the reexcitation of the antenna chlorophylls and not giving rise directly to a fluorescence emission.

Current models for the origin of the different decay components are based on the α/β type heterogeneity of PS II (see, for example, Refs. 16, 21). However, it is difficult to explain the results presented in this work in these terms, based on the proposed location and organisational properties of the two forms of PS II. For instance, only PS II_α have been found in PS-II-enriched membranes [20], and PS II_α are connected units while PS II_β are isolated centres [16]. If this latter point is the case, then it is expected that PS II_α would produce component(s) showing a proportional change in both lifetime and yield on PS II closure (as seen in Figs. 2 and 3), while PS II_β would have different lifetime(s) for open and for closed centres which only change in yield on closing PS II (see Ref. 24). This type of trend is not seen in this work. The fact that the two long-lived variable components observed in the PS II particles are similar to those found in the intact photosynthetic system [5,8] suggests that they have the same origins in the two cases. Holzwarth et al. [6] have proposed that, in vivo PS II_β is identified by a 500 ps decay at F_0 and a 1200 ps decay at F_m whereas PS II_α has a lifetime of 200 ps at F_0 and 2 ns at F_m. This conclusion is based mainly on lifetime and amplitude data at the two extreme levels of fluorescence and infer that the two types of centre are not

connected, which is contrary to the original definition of PS II_{α} centres. These lifetimes are equivalent to those observed in the PS-II-enriched membranes, in which the least-squares regression analyses of the datum points show that it is the 150 ps decay which becomes the 900 ps component and that the 350 ps decay becomes the 2 ns component. This suggests that none of the major variable PS II decays can be explained by an isolated system and that, contrary to the conclusions in Refs. 2 and 6, the components observed can not be explained by a PS $II_{\alpha/\beta}$ model and neither by the proposal that the 500 ps component becomes the 1.2 ns decay and the 200 ps component gives rise to the 2 ns decay at F_m.

It has been suggested previously that there is a rapid PS II component [6,8,10]. It can be seen from Fig. 2 that the rapid PS II component is infact variable in nature and that there is a rapid PS II decay still present at F_m, which is again contrary to the recent proposals of Holzwarth and co-workers [2,6]. An interesting observation is that this rapid component gives rise to lifetimes at F_0 and F_m similar to those exhibited by isolated chromatophores of *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* [26]. This emission probably corresponds to antenna pigments associated directly with the reaction centre proteins. This conclusion is based on (i) preliminary modelling studies using the bipartite and tripartite models of Butler (see Refs. 14,15) in which the fastest decay is determined solely by the given rate of photochemistry, and (ii) preliminary lifetime measurements on PS-II-core-complex preparations containing very little LHC II in which 80% or more of the relative amplitude is associated with components with lifetimes of less than 300 ps (not shown).

The slower components probably arise from the peripheral antenna system which give rise to lifetime values governed mainly by the energy transfer rates between neighbouring antenna proteins and the probability of finding an open PS II reaction centre. These two decays might be indicative of the recently proposed heterogeneous population of LHC II (see Ref. 27). Based on observations already made in which a double mutant of *Chlamydomonas reinhardtii* lacking PS I and PS II exhibits two components, with lifetimes of 2.7 ns and 0.9 ns [7], and isolated LHC II which produces two lifetime components of 3.4 ns and 1.0 ns [28], such a suggestion does not seem improbable.

With respect to the 'intact' thylakoid membrane system, we suggest that the two, slower variable decays seen in the PS II particles correspond to the two variable in vivo components. It appears that in vivo the rapid PS II variable emission is not fully resolved due to the presence of constant rapid PS I emissions having lifetimes similar to those of the PS II component (see Refs. 6,8,9,28) which mask its true variable nature. Another important consequence of our observations is that F_0 is not a constant fluorescence emission and in vivo it contains the three variable PS II emissions along with constant PS I emission(s). When measuring the steady-state fluorescence induction curve, part of the F_0 becomes the variable fluorescence and therefore certain lifetime components will not be present at F_m. This observation has grave consequences for the significance of the conclusions put forward in Ref. 13. In that work, the F_0 decay was subtracted from the F_m decay to produce the 'F_v' decay, On analysing this decay, it was seen that a lag component was required for the best fit, which was taken as evidence for the charge recombination hypothesis for variable fluorescence. It can be seen that, due to the variable nature of the PS II components at F_0, several of the rapid decays will be absent from the F_m decay, leading to negative components after the subtraction. Therefore, this piece of evidence supporting the luminescence hypothesis for variable fluorescence is not valid.

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