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Time-resolved picosecond fluorescence spectra of the antenna chlorophylls in *Chlorella vulgaris*. Resolution of Photosystem I fluorescence *

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The time-resolved fluorescence emission and excitation spectra of *Chlorella vulgaris* cells have been measured by single-photon timing with picosecond resolution. In a three-exponential analysis the time-resolved excitation spectra recorded at 685 and 706 nm emission wavelength with closed PS II reaction centers show large variations of the preexponential factors of the different decay components as a function of wavelength. At $\lambda_{em} = 685$ nm the major contribution to the fluorescence decay originates from two components with life-times of 2.1–2.4 and 1.2–1.3 ns. A short-lived component with life-times of 0.1–0.16 ns of relatively small amplitude is also found. When the emission is detected at 706 nm, the short-lived component with a life-time of less than 0.1 ns predominates. Time-resolved emission spectra using $\lambda_{exc} = 630$ or $\lambda_{exc} = 652$ nm show a spectral peak of the two longer-lived components at about 680–685 nm, whereas the fast component is red-shifted as compared to the others and shows a maximum at about 690 nm. The emission spectrum observed upon excitation at 696 nm with closed PS II reaction centers shows a large increase in the amplitude of the fast component with a lifetime of 80–100 ps as compared to that at 630 nm excitation. At almost open Photosystem II (PS II) reaction centers (F_0), the life-time of the fast component decreased from 150–160 ps at 682 nm to less than 100 ps at 720 nm emission wavelength. We conclude that at least two pigment pools contribute to the fast component. One is attributed to PS II and the other to Photosystem I (PS I). They have life-times of approx. 180 ps and 80 ps, respectively. The 80 ps (PS I) contribution has a spectral maximum slightly below 700 nm, whereas the 180 ps (PS II) spectrum peaks at 680–685 nm. The spectra of the middle decay component τ_m and its sensitivity to inhibitors of PS II suggest that this component is not preferentially related to LHC II but arises mainly from Chl *a* pigments probably associated with a second type of PS II centers. The amplitudes of the fast (180 ps, PS II) component and the long-lived decay show an opposite dependence on the state of the PS II centers and confirm our earlier conclusion that the contribution of PS II to the fast component probably disappears at the F_{max} state (Haehnel W., Holzwarth, A.R. and Wendler, J. (1983) Photochem. Photobiol. 34, 435–443). Our data are discussed in terms of α,β-heterogeneity in PS II centers.

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Abbreviations: LHC, light-harvesting complex; Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS, Photosystem; F_0, fluorescence level at fully open PS II centers; F_{max}, fluorescence level at fully closed PS II centers; F_{var}, variable fluorescence

Introduction

In green plants and algae light energy is absorbed by a large array of light-harvesting chlorophyll (LHC) *a/b* proteins and by chlorophyll (Chl) *a* proteins associated with the reaction centers of either PS I or PS II. Still a great deal of uncertainty exists on the detailed organization and function of the photosynthetic apparatus and the processes relevant for the first steps in photosynthesis after absorption of a photon. Time-resolved fluorescence spectroscopy provides a valuable tool for investigating these processes. It has been recognized that single-photon timing combined with picosecond laser excitation is the method of choice for studying the fluorescence decay in photosynthetic systems at low excitation intensities [1] (for recent reviews, see Refs. 2 and 3). It now provides excellent sensitivity with a time-resolution of better than 20 ps [4]. Furthermore, it has great potential for resolving complex multicomponent decays. This latter possibility is of considerable importance in view of the complexity of the photosynthetic apparatus. Different laboratories agree with respect to the general features of the decay kinetics in chloroplasts and green algae found by this technique [5–9]. In general, three decay components are required to describe the in vivo fluorescence decay of Chl pigments. Their amplitudes and lifetimes respond differently to the redox state of PS II. A fast component of 80–150 ps lifetime has been ascribed to open PS II centers [5,6,8]. An intermediate component of several hundred ps was attributed mainly to LHC II [5,8,10] or, alternatively, to PS II in a different redox state [6]. A long-lived decay component of 1–2 ns was recognized as being related to the amount of closed PS II centers. Its amplitude is zero at fully open centers [8] (F_0 state) and maximum when all PS II centers are closed either by light [5,10] or inhibitors of PS II [8] (F_{max} state). It has been concluded that this component was almost exclusively responsible for the well-known variable fluorescence (F_{var}) observed upon closing PS II centers [5,8,10]. These basic features are now fairly well established. In order to get more detailed information picosecond investigations were performed by varying external conditions as, e.g., cation concentration [10], redox potential [11,12] and the phosphorylation state of the membrane proteins [13] or were carried out on thylakoid fragments [14]. These experiments gave more insight into the complexity of the system but did not provide an answer to some of the most basic problems. Does the middle component really originate from LHC II? What is the contribution of PS I fluorescence at room temperature? The latter has never been resolved in intact cells. Does the amplitude of the fast PS II component disappear at the F_{max} state, as suggested by us [8,14], but not observed by other workers? These and many other questions remain to be answered.

Measuring the time-resolved emission and excitation spectra of the various decay components should provide a powerful method to approach these problems. By this way the characteristics of the emitting pigments, the excitation and/or absorption spectra of the connected antennae, as well as the number of kinetic components involved, might be resolvable. Variation of other parameters as, e.g., the redox state of PS II, may provide more insight into the origin of these individual decay components.

Following this line we have been able to distinguish four different fluorescence contributions. A newly resolved component arises from PS I with a spectral emission maximum slightly below 700 nm. The time-resolved spectra also indicate that the middle component does not arise from LHC II fluorescence as supposed previously [5,8,10], but should probably be assigned to a second type of PS II centers. The spectra of the individual decay components obtained in this way are consistent with steady-state spectra of intact photosynthetic membranes, thylakoid fragments, and isolated pigment proteins. A preliminary account of this work has been presented in Ref. 15.

Materials and Methods

Chlorella vulgaris, strain 221-11b has been grown as described [8]. The algae were harvested during the logarithmic growth phase and washed once with nutritional medium. For the measurements the algae were diluted with 50 mM Tris-sulfate buffer (pH 8.0) containing 20 mM $NaHCO_3$ to give a concentration of 10 μg Chl/ml. The chlorophyll concentration in the algae suspension was

determined after extraction with methanol at 55°C as described [16]. For closing PS II reaction centers (F_{max}) 20 μM DCMU and 10 mM hydroxylamine hydrochloride, adjusted to pH 7.0, were added to the algae suspension 15–20 min before the measurements and the samples were preilluminated with white light of low intensity to close the PS II centers. The samples were pumped through a 3 × 3 mm flow cuvette at rates up to 300 ml/min in the case of fluorescence decays with open reaction centers and at (3–5)-times lower rates at closed reaction centers. All measurements were carried out at room temperature (21–24°C). The laser beam had a diameter of approx. 1 mm.

Time-resolved fluorescence measurements were performed using a synchronously pumped and cavity-dumped dye laser system with a mode-locked argon ion laser (Spectra Physics) as the pumping source. This system emits light pulses of typically 10 ps duration at variable repetition rates. The dye laser was operated using DCM dye with a tuning range of 610–730 nm. The detection system was a single-photon timing apparatus. Fluorescence was selected by a double-monochromator with slits set to give a 4 nm bandwidth. A red-sensitive photomultiplier (R955, Hamamatsu) with a multialkali photocathode was used for detection. A full width at half maximum of less than 130 ps and a full width at tenth maximum of 240 ps was measured by single-photon timing for the excitation function. The high sensitivity of the single-photon timing technique enabled us to use laser pulse intensities for excitation of less than $1 \cdot 10^{10}$ photons/cm^2 at a repetition rate of 800 kHz. Life-times according to multiexponential kinetics were calculated by an iterative convolution method using a semilinear Marquardt-algorithm. The quality of the fits was judged by both a reduced χ^2 criterion and a plot of weighted residuals [17]. A fitting range of 6 and 10 ns has been used for decays at the F_0 and F_{max} states, respectively.

In general, a model function comprising only three exponentials could be fit to these data when all parameters were free-running. However, many of the three-exponential fits were not ideal as judged from the residuals, indicating the requirement for more than three components. From other work [4] we are confident that the deviations in the residuals are not caused by instrumental artifacts. Although four-exponential analysis with free-running parameters was possible only on a few of the measured decays, we tried to resolve the short-lived component further by the procedures described in the following: when a decay curve composed of four exponential components is deconvoluted by a model function containing three exponentials, the calculated decay times will represent some averaged values of the real decay times. Which life-times will be mixed to the largest extent depends on the relative amplitudes and relative lifetime values of the real components. Our data suggest that the shortest-lived component of the three-exponential analysis is composed of two decays. We can write the following relationships:

$$A_1\tau_{f'} + A_2\tau_{f''} = (A_1 + A_2)\tau_f = A_f\tau_f \quad (1)$$

which represents conservation of the fluorescence yields ϕ, i.e.:

$$\phi_1 + \phi_2 = \phi_{tot}$$

and:

$$\frac{A_1\tau_f' + A_2\tau_{f''}}{A_1 + A_2} = \tau_f \quad (2)$$

which represents the averaging of the decay times of the two components. In the above equations A_i and τ_i denote absolute amplitudes and lifetimes, respectively, τ_f denotes the averaged fast component from the three-exponential analysis. This relationship holds for pulse experiments when the life-times are not considerably shorter than the half-width of the apparatus function.

For our time-resolved spectra analysis we are interested in the amplitudes A_1 and A_2 of the unknown components. They can be estimated from the nonresolved experimental values A_f and τ_f using Eqns. 1 and 2 once we have fixed the values for $\tau_{f'}$ and $\tau_{f''}$. In order to test the validity of this approach we have carried out a number of simulations. For a typical decay measurement with mostly open PS II centers the results of a simulation are shown in Fig. 1. Four exponentials with life-times 80, 180, 800 and 1500 ps have been added with the amplitudes given in Table I. The resulting simulated decay curve has been convoluted with an excitation function modeling reasonably our ap-

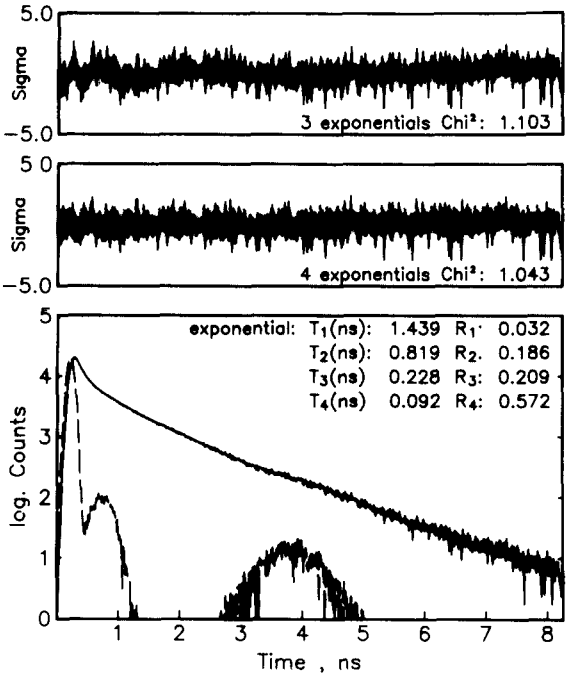

Fig. 1 Simulated four-exponential fluorescence decay for testing the resolution capabilities of our deconvolution technique. The results shown in the inset are those from a four-exponential fit with free-running parameters.

Note Figs. 1–3 The lower panel shows on a semilogarithmic scale the measured (or simulated) fluorescence decay (full line), the excitation pulse (broken line) and the results of the deconvolution procedure (inset; $T_1 .. T_n$ are life-times and $R_1 ... R_n$ are relative amplitudes). The upper panels show the residual plots and χ^2-values for deconvolutions using model functions with 2–4 exponential components.

paratus function. Poissonian noise was then added to both the decay and the excitation function and the deconvolution was performed as usual. The theoretical and deconvoluted simulated data are given in Table I. For a three-exponential model function a slight systematic deviation in the residuals plot is observed (cf. Fig. 1). Using the method described above, amplitudes of 44.1 and 28.2% were calculated for the two fixed lifetime components of 80 and 180 ps, respectively (cf. Table I). This shows that the resolution of the two fastest decay components from the three-exponential deconvolution recovered the original data fairly well (cf. Table I). It should be pointed out that under these conditions there will occur some systematic underestimation of the amplitude of the fastest component. The extent of this underestimation is

TABLE I

DATA OF THE SIMULATED DECAY SHOWN IN FIG. 1

All lifetimes τ are given in ns and all amplitudes A in %. Column 1 gives the input values used for the simulation of the four-exponential decay. Columns 2 and 3 give the results of a four- and three-exponential deconvolution, respectively, with free-running parameters. Column 4 gives the results of the resolution of the fast component from a three-exponential fit according to the algebraic procedure described in Material and Methods. Column 5 gives the results of a four-exponential fit using two fixed life-times of 80 ps and 180 ps and two unconstrained life-times.

τ_1	0.08	0.092	–	0.08 [a]	0.08 [a]
A1	44	57	–	44	43
τ_2	0.18	0 228	0.119	0.18 [a]	0 18 [a]
A2	33	21	73	28	34
τ_3	0 8	0.82	0.64	0 64	0.82
A3	20	19	19	19	20
τ_4	1 5	1.44	1.22	1.22	1 51
A4	3	3.2	8	8	3

[a] Fixed lifetimes used for the resolution.

small, however, as compared to the other errors. Also the middle and long-lived components are affected slightly by this approach. However, a four-exponential deconvolution of our test data with all parameters free-running did not yield better results. This shows that decay analysis is limited by statistical factors.

In a second approach we applied a computer program that allowed us to put constraints on some of the lifetimes. In our case we held the two short lifetimes constant, while the other two as well as all four preexponential factors (amplitudes) were free-running. By this way only six (two lifetimes and four amplitudes) instead of eight parameters had to be determined in a four-exponential analysis. Using this approach all our experimental decays could be analyzed in a four-exponential fit. One may expect that in this case the amplitudes of the fixed life-times are determined with better accuracy than in the more simple approach using method 1. However, the requirement to determine the values of the fixed life-times beforehand still remains. We found that the two methods gave the same result within the error limits, except for the slightly higher (by approx. 5–10%) amplitude of the fastest component obtained with method 2.

This does not change any of our conclusions. The experimental example given in Fig. 3 shows the close resemblance between the experimental and simulated data (Fig. 1).

All calculated amplitudes have been normalized to a constant measuring time and constant exciting light intensity. For correction of time-resolved spectra the light intensity has been measured using a calibrated radiometer (model G 550 –1 EG + G Ortec). The amplitudes of the various decay components in the excitation spectra are thus proportional to the absorption cross-section for that particular component. The time-resolved fluorescence emission spectra have also been corrected for wavelength-dependent sensitivity of the photomultiplier/monochromator combination. For recording the correction function we used a calibrated iodine-quartz lamp (6.6 A, 200 W, Osram) and a calibrated diffuser (EG + G Ortec). The lamp was operated with a constant current power supply. Calibration data were provided in 5 nm intervals. The calibrated diffuser was positioned in place of the sample and it was illuminated by the lamp positioned at a distance of approx. 60 cm. The emission spectrum of the lamp was then recorded by scanning the emission monochromator under conditions identical to those used in the life-time measurements.

Results

The fluorescence decay of *Chlorella vulgaris* monitored at 674 nm ($\lambda_{exc} = 652$ nm) at closed PS II reaction centers (F_{max}) is shown in Fig. 2. The residuals plot indicates that a fit with a sum of two exponentials is not sufficient to describe the decay curves. Instead a fit with a sum of at least three exponential functions is required. An analysis of the fluorescence decay of *Chlorella vulgaris* excited at $\lambda_{exc} = 630$ nm and detected at $\lambda_{em} = 686$ nm at a state close to F_0 is shown in Fig. 3. The frames on top of Fig. 3 show the residual plots for fits

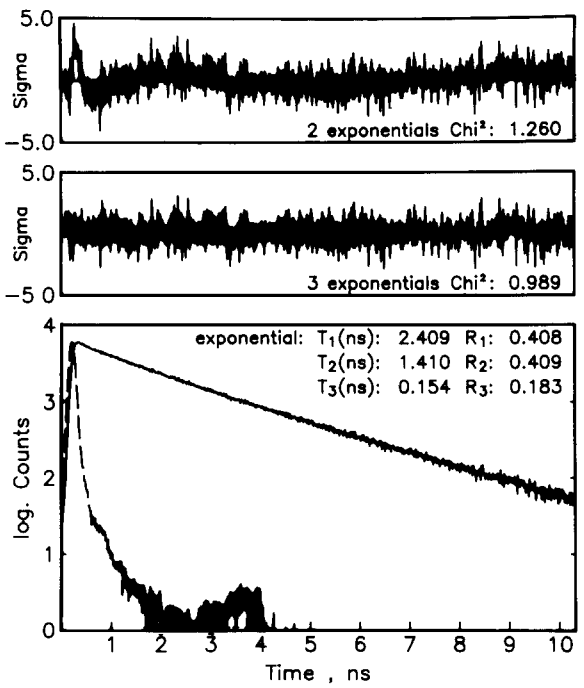

Fig 2 Fluorescence decay of *Chlorella vulgaris* in the F_{max} state (DCMU and hydroxylamine added and preilluminated); λ_{exc} = 652 nm, λ_{em} = 674 nm (cf. Note Fig. 1).

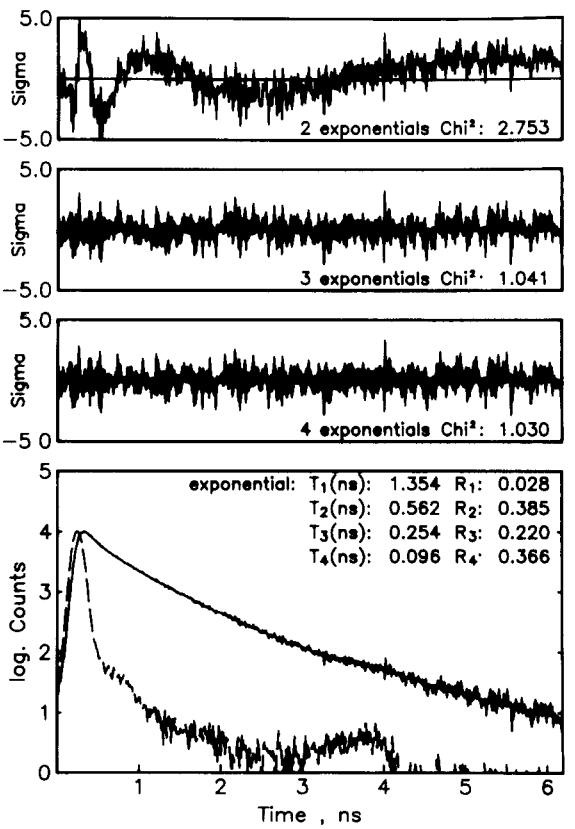

Fig. 3. Fluorescence decay of *Chlorella vulgaris* close to the F_0 state (no inhibitors added); $\lambda_{exc} = 630$ nm, $\lambda_{em} = 686$ nm; (cf. Note Fig. 1)

with a sum of two, three and four exponential components. It can be seen that a three-exponential fit is sufficient from a statistical point of view to describe the measured decay. However, a four-exponential fit with free-running parameters was also possible and it split the fast component into two contributions of 96 and 250 ps (cf. inset Fig. 3). A similar situation holds also for some of the other decays. However, such a four-exponential analysis was not possible for most of our data, and consequently all decays were first deconvoluted in terms of three exponential functions.

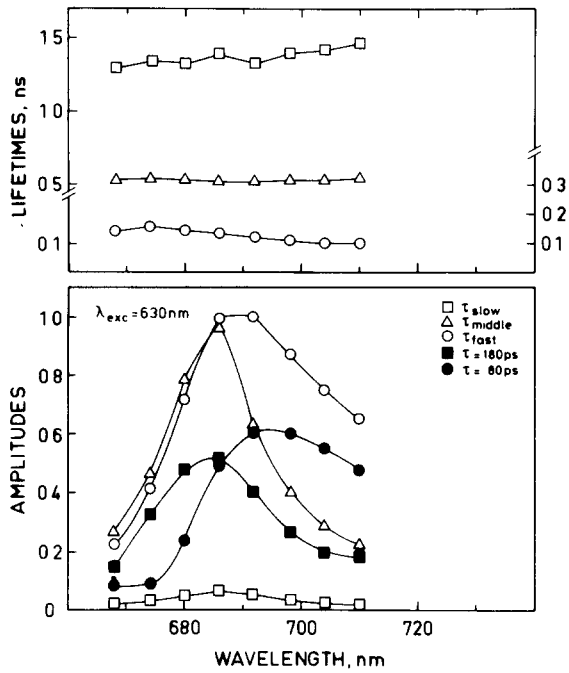

Fig. 4 Time-resolved fluorescence emission spectra of the individual decay components of *Chlorella vulgaris*; $\lambda_{exc} = 630$ nm. The algae were in a state close to F_0 (cf Results).
Note Figs. 4–10. Upper panel: fluorescence life-times of the individual decay components. Lower panel: absolute amplitudes of the individual decay components Open symbols denote the parameters that have been obtained directly from a three-exponential deconvolution of the decays. The closed symbols in the lower panel denote the amplitudes for the two resolved fast components calculated according to the procedure described in Materials and Methods These data have been calculated using the life-times and amplitudes of the deconvoluted fast component (open circles). In the upper panel (life-times) the scale at the right-hand side refers to the fast life-time component whereas the scale on the left-hand side refers to the two longer-lived decays

Time-resolved emission spectra for open PS II centers

Figs. 4 and 5 show the time-resolved emission spectra (amplitudes of time-resolved decay components) and their corresponding life-times of *Chlorella vulgaris* for excitation at 630 and 652 nm. At 630 nm and 652 nm Chl *a* or Chl *b*, respectively, were preferentially excited. No inhibitors were added for these measurements. Excitation light intensity was moderate and the samples were pumped at 300 ml/min. The fast component τ_f has a single amplitude maximum around 690 nm and a broad spectrum extending into the far-red region. Its life-time decreases from approx. 150–100 ps towards longer emission wavelengths. The other two components show a spectral maximum around 680–685 nm. For Figs. 4–10, the open symbols refer to the parameters calculated from the three-exponential fits. The closed symbols represent the resolution of the fast component into two components with fixed life-times according to the procedure outlined in the Materials and Methods section.

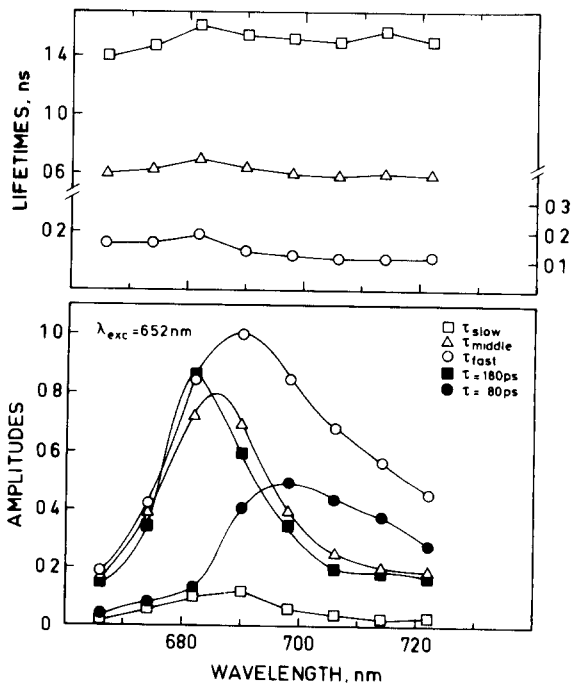

Fig. 5. Time-resolved fluorescence emission spectra of the individual decay components of *Chlorella vulgaris*; $\lambda_{exc} = 652$ nm. The algae were in a state close to F_0; (cf. Note Fig. 4)

Closed PS II reaction centers

At closed PS II reaction centers the time-resolved spectra given in Figs. 6–8 are obtained. The life-time τ_f of the fast component is generally shorter and the spectrum of this component spectrum is more red-shifted as compared to open PS II reaction centers. The life-time values of the middle component are nearly twice of those found in open PS II centers.

For excitation at long wavelength ($\lambda_{exc} = 696$ nm; cf. Fig. 8) the amplitude of the fast decay component is much larger than the one of the other components. The life-time is remarkably constant at 80–90 ps over that whole range. The spectrum decreases monotonically towards longer wavelength without showing a maximum. Both the middle and the long decay components have very small amplitude.

Time-resolved fluorescence excitation spectra

Time-resolved excitation spectra have been recorded at the emission wavelengths 685 (Fig. 9) and 706 nm (Fig. 10) for closed PS II reaction centers. The excitation spectra for open PS II

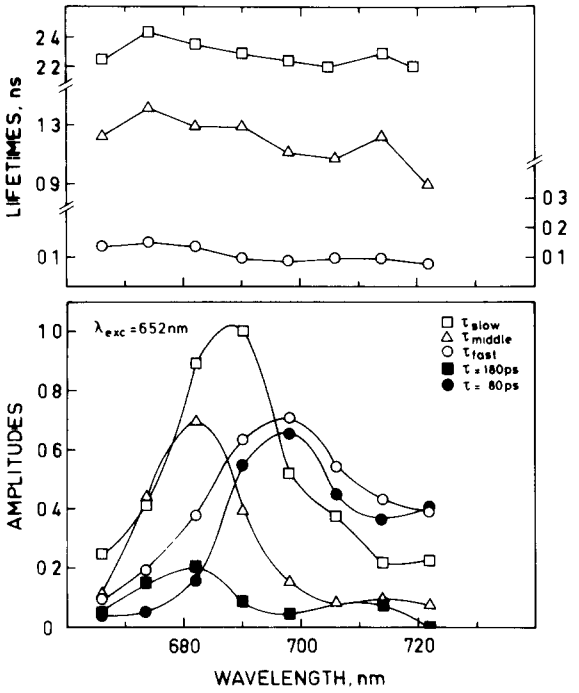

Fig 7. Time-resolved fluorescence emission spectra of the individual decay components of *Chlorella vulgaris*; $\lambda_{exc} = 652$ nm. The algae were in the F_{max} state, (cf. Note Fig. 4).

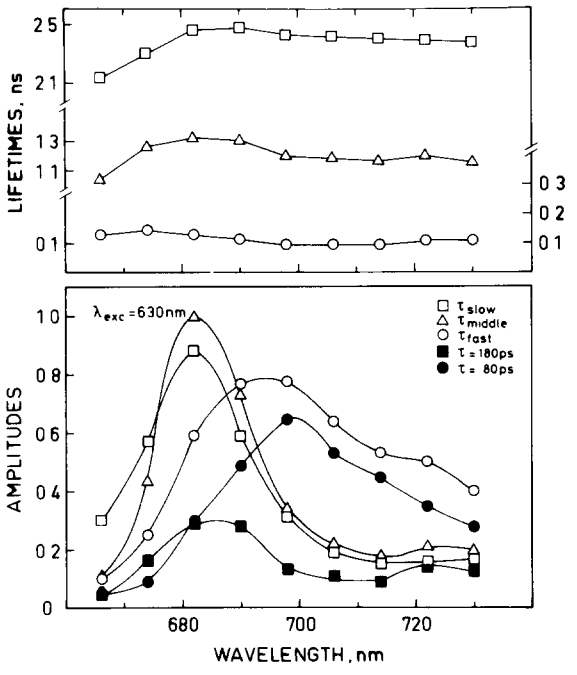

Fig 6 Time-resolved fluorescence emission spectra of the individual decay components of *Chlorella vulgaris*; $\lambda_{exc} = 630$ nm The algae were in the F_{max} state; (cf. Note Fig. 4)

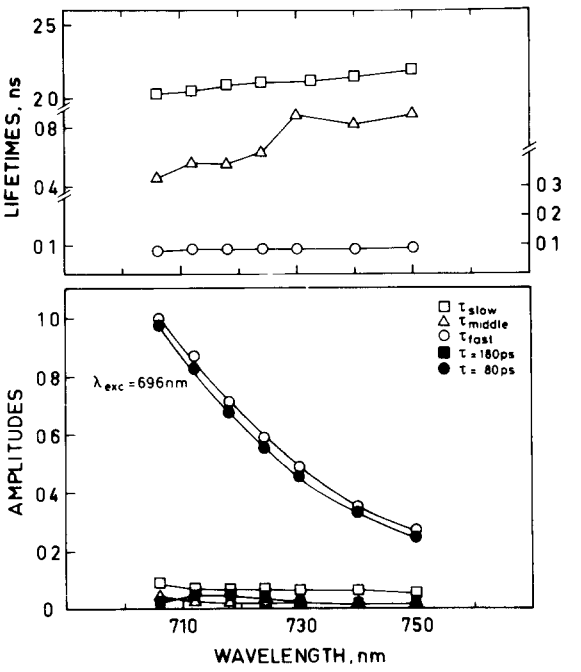

Fig. 8. Time-resolved fluorescence emission spectra of the individual decay components of *Chlorella vulgaris*; $\lambda_{exc} = 696$ nm. The algae were in the F_{max} state; (cf Note Fig. 4)

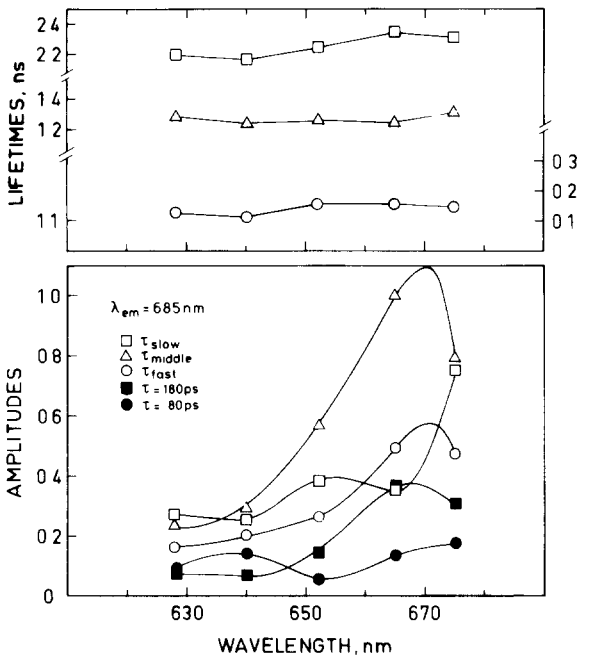

Fig. 9. Time-resolved fluorescence excitation spectra of the individual decay components of *Chlorella vulgaris*; $\lambda_{em} = 685$ nm. The algae were in the F_{max} state; (cf Note Fig. 4)

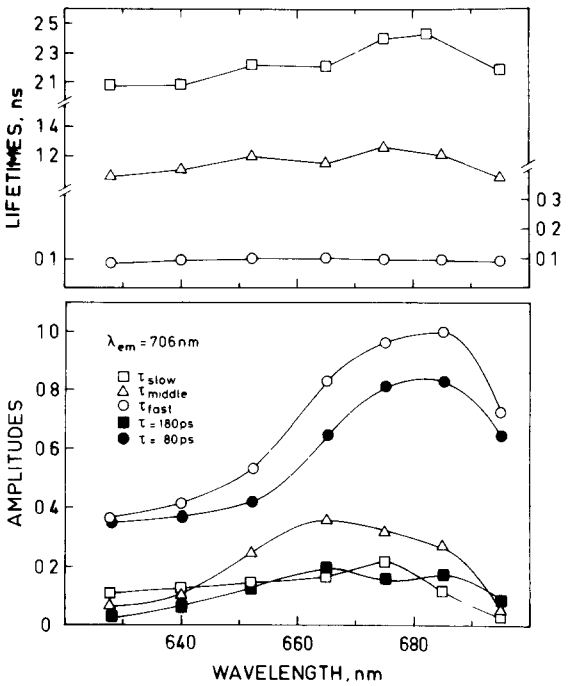

Fig. 10 Time-resolved fluorescence excitation spectra of the individual decay components of *Chlorella vulgaris*, $\lambda_{em} = 706$ nm. The algae were in the F_{max} state, (cf. Note Fig 4).

centers were not recorded because we could not keep the percentage of closed (by the excitation light) PS II reaction centers constant when changing the excitation wavelength. For $\lambda_{em} = 685$ nm (Fig. 9) the fast decay component has an excitation maximum around 670 nm and a second maximum around 635–640 nm. Its lifetime is shorter in the 630–640 nm region and longest at and above 650 nm. The middle component shows a single excitation band with a maximum at 665–670 nm. It has neither a maximum nor a shoulder at 652 nm but decreases monotonically to shorter wavelengths. The spectrum of the long-lived decay component is structured with maxima at 652 and around 675 nm and a minimum near 665 nm.

Fig. 10 shows the time-resolved excitation spectra recorded at the emission wavelength 706 nm. The spectrum of the fast component is now very broad and it is red-shifted as compared to $\lambda_{em} =$ 685 nm (Fig. 9). Its excitation maximum is around 685 nm and a strong shoulder appears at approx. 670 nm. There is neither a maximum nor a minimum at 652 nm. Its life-time is almost constant across the excitation band with values in the range 80–100 ps.

Discussion

Our data indicate two different types of life-time variations upon variation of external parameters. The first type occurs upon closing PS II reaction centers ($F_0 \rightarrow F_{max}$). These variations have been reported previously by ourselves in green algae [8] and inside-out vesicles (Ref. 14 and Haehnel, W., Wendler, J. and Holzwarth, A.R., unpublished data), and by Sauer and co-workers in Chloroplasts and algae [5,10,12] and by Berens et al. [7,9]. They will be discussed below.

The second type of lifetime variations is observed at a given state of the PS II reaction centers, when emission or excitation wavelength are varied. This refers to all three decay components, both at open and closed reaction centers. However, the relative variations are strongest for the fast decay component. Besides statistical errors there are a number of possibilities which could cause these variations.

Inspection of all the data recorded under the various conditions (wavelength dependence, DCMU-effect) indicates that these variations are not of statistical but rather systematic nature. They are intimately related to the state of PS II centers as well as to the excitation and emission wavelengths. We are aware of the problems related to the fitting procedure as well as of the possibility that a deconvoluted component might represent the superposition of two or more components. Our time resolution of about 15 ps due to our apparatus function of less than 130 ps full width a half maximum on the one hand, and the wavelength dependence of the lifetime τ_f on the other hand, make us confident to conclude that τ_f of our three-exponential analysis results from two different components. In Materials and Methods we have described an approach to resolve these components (represented by closed symbols in Figs. 4–10). We have plotted the amplitudes of the various decay components rather than their yields, because the former are directly proportional to the absorption cross-sections.

The fast decay component(s)

In the three-exponential analysis the fast decay component has the largest value of its life-time (180–190 ps) at $\lambda_{em} = 682$ nm at open PS II reaction centers (F_0) and excitation into the Chl *b* absorption band at 652 nm. Closing the PS II reaction centers by inhibitors (F_{max}) and/or changing the excitation or emission wavelengths, in particular to the red region, causes a decrease in this lifetime. The lowest life-time values are obtained at closed PS II reaction centers at $\lambda_{em} = 700$ nm and excitation at 630 or 696 nm. The time-resolved emission spectra of this component are broader and shifted to longer wavelengths at the F_{max} state as compared to the F_0 state.

All of these findings indicate an inhomogeneous origin of the fast component τ_f. Therefore we have resolved this component of variable decay time into two contributions with constant life-times of 80 ($\tau_{f'}$) and 180 ps ($\tau_{f''}$). We have chosen these values because they represent approximately the shortest and longest values obtained by deconvolution for the fast τ_f decay under the various conditions. The spectra of the two subcomponents obtained in this way show interesting features (closed symbols in Figs. 4–10). At open PS II centers (F_0) the 180 ps subcomponent ($\tau_{f''}$) shows an emission maximum at 682–685 nm (cf. Figs. 4 and 5). Its relative amplitude is larger when excited at 652 (preferentially Chl *b* excitation) as compared to excitation at 630 nm (preferentially Chl *a* excitation). This latter observation indicates that the calculated 180 ps ($\tau_{f''}$) subcomponent represents fluorescence from Chl *a* molecules in close contact to pigment proteins with a high Chl *b*/Chl *a* ratio, i.e., the LHC II complex. We thus assign this decay component to antenna pigments of PS II in open reaction centers, comprising LHC II and Chl *a* II. This interpretation does not necessarily imply that the corresponding fluorescence spectrum arises from LHC II directly, since the LHC II → Chl a_{II} transfer time may be very short. It only means that the excitons are mostly created in the LHC II pool. The life-time of this component is probably determined by the charge separation and/or trapping kinetics in the PS II centers. In the F_{max} state the amplitude of this decay component decreases considerably as compared to the F_0-state. This is in agreement with our previous observations [8,14].

It is interesting that in the F_{max} state the efficiency of excitation for the remaining part of this 180 ps component at $\lambda_{exc} = 652$ nm and $\lambda_{exc} = 630$ nm is reversed as compared to the F_0 state. Now 630 nm excitation is more efficient that 652 in contrast to the situation in the F_0 state. This observation might indicate the presence of still another subcomponent with different origin than the one responsible for the fast component at open PS II reaction (mainly Chl *a* antenna) centers. In contrast to the fast PS II component this subcomponent does not disappear at the F_{max} level. The actual life-time of this component might of course differ from 180 ps, since this is the value which we assumed for the LHC II connected component derived from open PS II centers. An unequivocal assignment of this component is not possible at present.

We will now turn to a discussion of the 80 ps ($\tau_{f'}$) subcomponent. Its emission spectrum is strongly red-shifted with a maximum slightly below 700 nm under all conditions. The peak intensity does not depend, within the error limits, on the state of the PS II centers. Under most condi-

tions its spectrum exhibits a shoulder and sometimes a second maximum around 715–700 nm. Its amplitude at 682 nm is low. These features indicate that this subcomponent should be attributed to Chl *a* outer and core antenna of PS I [18]. Its main emission maximum situated slightly below 700 nm ideally fits the energy of the P-700 reaction center. The shoulder or maximum around 715–720 nm is probably due to PS I-Chl complexes different from the ones emitting close to 700 nm. They might rather be identical with those pigments that give rise to the strong low temperature emission in algae around 715–720 nm [19,20]. This emission is generally broad, in agreement with our room temperature time-resolved spectrum. Our time-resolved spectra for the fast component(s) at closed PS II reaction centers are in fact consistent with the spectrum observed for PS I particles at room temperature by Kyle et al. [21]. We have previously reported biexponential lifetimes of 30–40 ps and 120–130 ps in PS I particles (stroma laemllae fraction) [14] at $\lambda_{em} = 682$ nm. More recent, measurements at $\lambda_{em} = 706$ nm gave a lifetime near 80 ps (Haehnel, W., Wendler, J. and Holzwarth, A.R., unpublished results). Lifetimes of about 70 ps [22], 60 ps [23] and around 100 ps [24] have been reported for PS I particles isolated under various conditions. Our in vivo results reported here are in good agreement with these data. Thus our analysis allows the contributions of PS I to the room temperature fluorescence of algae to be accounted for in an overall consistent manner. Contributions of PS I to room temperature fluorescence of intact thylakoids and in particular to the fast decay component have not been resolved before, although it was assumed that they should contribute to a significant extent to the measured fluorescence decay [5,6,8,14]. It is interesting to note that our spectra indicate that only a small amount of quanta absorbed by Chl *b* contribute to the PS I component of 80 ps life-time. This is consistent with the finding that nearly 80% of Chl *b* is associated with PS II [25].

Finally, we should discuss whether and to what extent the choice of the two fixed short life-times influences our results. From our procedure used to determine these life-times it follows that the fast PS II lifetime (180 ps) probably represents a lower limit to the real value. The deviations could be up to 50 ps. This would change the shape of the resolved spectra only slightly and it would not change our conclusions. The important basic features of the kinetics can be resolved readily using our approach.

The middle component

Fig. 4–7 indicate that the middle component is more efficiently excited at $\lambda_{exc} = 630$ nm than at $\lambda_{exc} = 652$ nm. Its amplitude is invariant on the redox state of the PS II centers. However, its life-time changes from 0.5–0.6 ns at the F_0-state to 1.0–1.4 ns at the F_{max} state, i.e., by a factor of 2–3. The excitation spectra indicate that these pigments have maximal absorption near 670 nm and low Chl *b* content (no maximum or shoulder at 652 nm). The excitation spectrum of this component with $\lambda_{em} = 685$ nm looks like a typical Chl *a* spectrum. This excludes a preferential origin from LHC II, in contrast to what has been supposed earlier [5,8,10]. The emission peaks around 682–685 nm with a tail towards longer wavelengths. On the basis of its DCMU/NH_2OH sensitivity this component should be assigned to PS II centers. However, its emission spectrum and, in particular, its excitation spectrum indicate that these PS II centers carry a different antenna than those that give rise to the 180 ps component at F_0. They also differ in other properties. It is this component that makes the highest contribution to the F_0 fluorescence. Furthermore our data show that the 0.5–0.6 ns component at F_0 and the 1.0–1.4 ns component at F_{max} correspond to each other and originate from the same pigment pool(s). The broad excitation spectrum might indicate that several pigment pools contribute to it.

The long-lived component

In agreement with earlier reports [5,8,10] the amplitude of the long-lived component is found to be related to the redox state of PS II reaction centers. Its amplitude is maximal when all PS II centers are closed and approaches zero when all are open [6,8]. In this work it was not our main aim to reach the state of fully open PS II centers. The very low excitation intensity required for achieving that situation would have been in conflict with our intention to measure time-resolved spectra over a wide emission wavelength range.

This leads to very long measuring times at the long- and short-wavelength edges. Instead we have chosen a state of mostly open PS II centers, as incidated by the relatively low amplitudes of the long-lived component of a few percent (Figs. 4 and 5). This small amount does not interfere with our above mentioned goal [8,14]. Our data show that the long-lived and the short-lived components of PS II originate from the same pigment pool. This confirms our earlier conclusions [8,14]. The excitation spectrum of the long-lived component with closed PS II centers $\lambda_{em} = 685$ nm (Fig. 9) shows a Chl *b* spectrum with a minimum at 665 nm. We assign this component to pigments energetically coupled to LHC II. At open PS II reaction centers the same conclusion can be drawn for the 180 ps PS II component from a comparison of the relative efficiencies for excitation at $\lambda_{exc} = 630$ nm and 652 nm (Fig. 4 and 5). This agreement in the excitation efficiencies of the short and long-lived decays, together with the observed opposite changes in their amplitudes, provides further support to our previous interpretation that at each of the two extreme states F_0 and F_{max}, respectively, one of the two components has very small or even zero amplitude. This is further supported by the fact that the amplitude of the long-lived component at F_{max} and that of the fast PS II subcomponent $\tau_{f''}$ (180 ps) at F_0 are approximately equal.

PS II heterogeneity

Our data show that in the F_0 state the fast subcomponent ($\tau_{f''} = 180$ ps) and the middle component ($\tau_m = 0.5$–0.6 ns) should be assigned to PS II. In the F_{max} state the different spectra of the slow ($\tau_s = 2.1$–2.4 ns) and middle ($\tau_m = 1.1$–1.4 ns) components and their life-times in comparison to the F_0 state indicate that they should both be assigned to PS II, though to reaction centers of different types. We can also conclude that upon closing PS II reaction centers the fast component turns into the slow phase while the middle components in the open and closed states correspond to each other in a similar manner. This points to a basic heterogeneity in PS II. Thus, in order to explain our results, a model of the photosynthetic apparatus is required that accounts for this heterogeneity. Based mainly on fluorescence induction measurements [26–31] and redox titrations one such model has been proposed several years ago, and it is known as α,β-heterogeneity in the literature. Although somewhat controversial in the details the basic features of this PS II heterogeneity are widely accepted at least for higher plants [32]. Data on green alga are very sparse. According to the current understanding the α-centers should be located in the stacked regions of the thylakoids and they are connected to the LHC II pool [33–35]. The β-centers are believed to carry a smaller antenna. They should be located in the stroma-exposed regions of the thylakoids. Many aspects of the time-resolved spectra reported here could be reconciled with this type of PS II heterogeneity. Only the relative intensities of the components do not seem to be in full agreement with the expectations [32], although the available data refer to higher plant chloroplasts. We note that, on the basis of model calculations, Butler et al. [36] have proposed α,β-heterogeneity to explain our recent fluorescence decay data for *Chlorella* [8]. If the concept of α,β-heterogeneity should apply to our data the relationships indicated in Table II would be invoked. Another type of PS II heterogeneity, indicating differences in redox potentials, has been reported in the literature [28,37–39] and has also been studied by fluorescence kinetics [12]. Although we are strongly biased towards α,β-heterogeneity to rationalize our data it is presently not possible for us to decide unequivocally whether the observed PS II heterogeneity is related to one or more of the known differences in PS II centers discussed above or whether we are dealing with an entirely different phenomenon. In any case the two types of PS II centers differ in their efficiency of charge separation at F_0 and in their efficiency

TABLE II

SUGGESTED RELATIONSHIP BETWEEN DECAY COMPONENTS AT OPEN AND CLOSED PS II REACTION CENTERS BASED ON THE CONCEPT OF α,β-HETEROGENEITY

	Open PS II reaction center (ps)	Closed PS II reaction center (ps)
PS II(α)	180	2200–2400
PS II(β)	500–600	1200–1400
PS I	80	80

as fluorescence quenchers at F_{max} as can be concluded from their lifetimes. These properties would also be consistent with α,β-heterogeneity [28,40,41].

Conclusions

The time-resolved spectra reported in this work provide three major results: firstly, the PS I fluorescence of intact cells has been separated from PS II fluorescence at room temperature. The spectra are in good agreement with the expectations based on thylakoid fragmentation. Secondly, we obtained evidence that the origin of the middle component, τ_m, has to be reinterpretated. This is in line with previous reports that in intermittent-light-grown chloroplasts which do not carry LHC II this middle component is also present [5,42]. Thirdly our data provide indications for a basic heterogeneity in PS II centers. Presently, we prefer the concept of α- and β-centers to rationalize this heterogeneity. More work is necessary, however, in order to clarify its origin fully. Finally, we have demonstrated that four different components are required to describe the decay kinetics in this system in general.

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