

Delayed fluorescence emitted from light harvesting complex II and photosystem II of higher plants in the 100 ns–5 μ s time domain

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Abstract This study presents the first report on delayed fluorescence (DF) emitted from spinach thylakoids, D1/D2/Cytb-559 preparations and solubilized light harvesting complex II (LHCII) in the ns time domain after excitation with saturating laser flashes. The use of a new commercially available multi-channel plate with rapid gating permitted a sufficient suppression of detector distortions due to the strong prompt fluorescence. The following results were obtained: (a) in dark-adapted thylakoids, the DF amplitudes at 100 ns and 5 μ s after each flash of a train of saturating actinic pulses exhibit characteristic period four oscillations of opposite sign: the DF amplitudes at 100 ns oscillate in the same manner as the quantum yield of prompt fluorescence, whereas those at 5 μ s resemble the oscillation of the μ s kinetics of P680⁺ reduction in samples with an intact water oxidizing complex, (b) the quantum yield of total DF emission in the range up to a few μ s is estimated to be $< 10^{-4}$ for thylakoids, (c) the DF of D1/D2/Cytb-559 exhibits a monophasic decay with $\tau \approx 50$ ns, (d) DF emission is also observed in isolated LHCII with biphasic decay kinetics characterized by τ values of 65 ns and about 800 ns, (e) in contrast to thylakoids, the amplitudes of DF in D1/D2/Cytb-559 preparations and solubilized LHCII do not exhibit any oscillation pattern and (f) all spectra of DF from the different sample types are characteristic for emission from the lowest excited singlet state of chlorophyll *a*. The implications of these findings and problems to be addressed in future research are briefly discussed. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Delayed fluorescence; Quantum yield; Photosystem II; Light harvesting complex; Water oxidizing complex

1. Introduction

Delayed fluorescence (DF) of photosynthetic organisms discovered by Strehler and Arnold [1] covers a wide time domain of many orders of magnitude (from ns to a few minutes) and depends essentially on three parameters: (i) the recombination rate of oxidizing (holes) and reducing (electrons) redox equivalents as a function of depth of their traps, (ii) the population of states with trapped electrons and holes and (iii) the quan-

tum yield of excited singlet state formation and radiative emission. Consensus exists that in oxygen evolving organisms DF is mainly emitted from photosystem II (PSII). Accordingly, monitoring DF provides a suitable tool to analyze the back reactions of trapped redox equivalents in PSII. Results and interpretations are summarized in several excellent reviews (see e.g. [2–4]). It has previously been proposed that the increase of fluorescence emission after ‘closure’ of PSII by keeping $Q_A^{\bullet-}$ in its reduced state is owing to fast DF caused by recombination of P680⁺Pheo⁻ [5]. Later detailed analyses led to the conclusion that P680 acts as a shallow trap for excited singlet states generated by light absorption of antenna pigments [6,7] and the excited singlet state ¹P680* is part of a rapid equilibrium with the radical ion pair P680⁺Pheo^{•-} [8,9]. Accordingly, the efficiency of photochemical charge separation is limited by the rapid electron transfer from Pheo^{•-} to Q_A which gives rise to a sufficiently stabilized state P680⁺Pheo $Q_A^{\bullet-}$. This process takes place with a rate constant of about (300 ps)⁻¹ [10–12]. Within the framework of the exciton (radical pair model [8,9]), a straightforward distinction between prompt fluorescence and DF cannot be achieved in the time domain of up to a few ns. Since the lifetime of the excited singlet state of chlorophyll (Chl) (¹Chl*) in solution is about 5 ns [13], this value provides a suitable criterion to distinguish kinetically between prompt fluorescence and DF.

The quantum yield of DF is rather small and has been estimated to be of the order of 10^{-3} compared with that of prompt fluorescence which is a few percent (for reviews, see [2–4]). Accordingly, DF measurements require a sensitive detector system. An additional problem arises for DF studies on the detrapping of holes from the water oxidizing complex (WOC) as a function of its redox states S_i . In this case, dark-adapted samples have to be excited with saturating flashes as a prerequisite for driving the Kok cycle of water oxidation at a minimum probability of damping (for review, see [14]). As a consequence, the sensitive detector system has to be protected from serious distortions caused by strong prompt fluorescence due to the actinic flash. This requirement can be easily satisfied by using mechanical shutters if the time gap between actinic flash and monitoring of DF is sufficiently long. However, the reduction of P680⁺ in samples with intact WOC is multiphasic with dominating ns kinetics (see [15] and references therein). Measurements of DF in this short time domain bear serious problems. In general, gated photomultipliers are used to avoid detector saturation. Rapid gating in the ns range is a difficult problem and therefore, up to now, only very few DF studies are reported for this time region [16,17]. The traces obtained, however, are of comparatively poor signal to noise ratio. Furthermore, the studies were performed with pre-reduced $Q_A^{\bullet-}$ or under repetitive flash excita-

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Abbreviations: Chl, chlorophyll; ¹Chl*, excited singlet state of Chl; DF, delayed fluorescence; LHCII, light harvesting complex II; MCP, multichannel plate; PSII, photosystem II; P680, Pheo and Q_A , photoactive chlorophyll, a pheophytin and a primary quinone acceptor, respectively, of PSII; S_i , redox states of the WOC; WOC, water oxidizing complex; Y_Z , redox active tyrosine of polypeptide D1

tion. Both approaches do not allow to study the S_i state dependent DF emission.

The development of a new generation of multichannel plate (MCP) detector units that permit very fast gating at high switching ratios opened a new road to address the problem of DF monitoring in the ns time domain after excitation of samples with strong actinic flashes. This communication presents the first report on results obtained with this new technique. It clearly shows that DF in the ns time domain not only originates from charge recombination in reaction centers but also includes contributions from reactions in the antenna system.

2. Materials and methods

2.1. Sample preparations

Thylakoid membranes and solubilized LHCII preparations were isolated from spinach by procedures described by Winget et al. [18] and Irrgang et al. [19], respectively. D1/D2/Cytb-559 complexes were prepared according to Seibert et al. [20] with slight modifications outlined in [21].

2.2. Equipment

Delayed light emission in the time window from 100 ns to 5 μ s after laser flash excitation was recorded with home-built equipment. The technical details of this apparatus are described elsewhere [22]. Here only a brief summary of the essential parts will be presented.

Samples were excited by a frequency doubled NdYAG laser (Spectrum GmbH, FWHM 10 ns, $\lambda = 532$ nm) at a repetition rate of 1 Hz and pulse energy of 0.4 mJ/cm². DF was monitored at an angle of 90° with respect to the incident laser beam. The emitted light was passed through a 532 nm laser blocker and guided to the MCP detector unit (R5916U-51, Hamamatsu) by means of light guide. This MCP can be rapidly gated ($\tau \approx 1$ ns) with a high switching ratio ($1:10^8$ at $\lambda > 500$ nm) and is equipped with a red-sensitive photocathode (multialkali element). Emitted photons were detected after wavelength selection with interference filters that were placed in front of the MCP. The signals were recorded with a digital storage oscilloscope (Tektronix TDS 520) and transferred via a GPIB-bus (PC IIA, National Instruments) to a computer for further data processing. The electrical band width was 100 MHz. Signal amplitudes were adjusted to a range between 1 and 20 mV by neutral density glass filter (NG5, Schott) in the optical pathway in order to avoid detector saturation. The overall time resolution of the set-up is limited by the jitter of the trigger device.

For measurements of flash dependent kinetics, the sample cuvette was flushed and refilled after each series of eight laser flashes with a home-built flow system. All measurements were performed at room temperature in a pH 6.5 buffer medium (50 mM morpholinoethane sulfonic acid, 10 mM NaCl). For LHCII and D1/D2/Cytb-559 preparations, the buffer medium contained 0.02% (v/v) *N*- β -dodecylmaltoside. Delayed light kinetics were recorded with a 695 nm interference filter (DAL 695, Schott) in front of the MCP. The spectral shape of delayed light emission was determined by using interference filters from Schott in front of the MCP.

Table 1

Target fit to triexponential kinetics with fixed lifetimes ($\tau_1 = 50$ ns, $\tau_2 = 300$ ns and $\tau_3 = 1.5$ μ s) plus a constant part of DF emission in dark-adapted spinach thylakoids excited with a train of four saturating laser flashes

Flash number	Amplitudes (rel. units)			
	a_1	a_2	a_3	a_4
1	11.6	0.62	0.28	0.04
2	8.2	0.59	0.28	0.08
3	8.9	0.46	0.31	0.11
4	9.1	0.66	0.30	0.09

The experimental data are shown in Fig. 1. The deconvolution into three decay components ('fast', 'middle' and 'slow') was checked by using independent fit procedures using free running parameters.

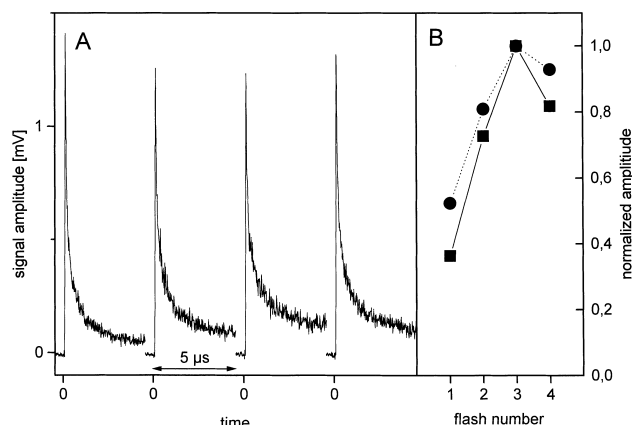


Fig. 1. Time course of DF emission from dark-adapted spinach thylakoids excited with a train of saturating laser flashes (A) and normalized DF amplitude at a delay time of 5 μ s (squares) and 7 μ s (circles, data gathered from [26]) as a function of flash number (B). The Chl content of the suspension was 10 μ g/ml, other experimental details as described in Section 2.

3. Results and discussion

Fig. 1 shows typical traces of DF emitted from dark-adapted spinach thylakoids within the time domain of 100 ns–5 μ s after excitation with a train of saturating laser flashes at a frequency of 1 Hz. The emission exhibits a characteristic dependence on the flash number and the overall decay is dominated by ns kinetics. The oscillation of the amplitudes in the flash sequence exhibits a period of four that is a 'fingerprint' for the reaction pattern of a functionally intact WOC. Interestingly, the amplitudes at 100 ns after the flash oscillate in a similar manner as the normalized yield of prompt fluorescence with maximum values after the first and fourth flash and smaller ones after the second and third flash [23,24]. The opposite feature emerges for the DF amplitudes measured 5 μ s after the actinic flash, DF (5 μ s). In this case, a pronounced maximum is observed after the third flash and the minimum value after the first flash. This pattern corresponds with previous results obtained in DF measurements of lower time resolution [25,26] as shown at the right side of Fig. 1. A few μ s after the actinic flash, PSII complexes with an intact WOC predominantly populate the state $Y_Z^{\text{ox}}P680Q_A^{\bullet-}$ because a large fraction of $P680^{\bullet+}$ is reduced by Y_Z via ns kinetics (see [27] and references therein) and the extent of $Q_A^{\bullet-}$ reoxidation is negligible ($\leq 5\%$) owing to the slow kinetics of both recombination with $P680^{\bullet+}$ [28,29] and electron transfer to Q_B (Q_B^-) (see [30] and references therein). Accordingly, the oscillation of DF (5 μ s) is ascribed to the S_i state dependent normalized extent of $P680^{\bullet+}$ reduction via μ s kinetics [27,31]. Analogously, it was expected that the DF decay in the ns time domain is also reflecting the corresponding kinetics of $P680^{\bullet+}$ reduction by Y_Z . A target fit deconvolution of the data into a triexponential decay plus a constant:

$$I_{\text{DF}}(t) = \sum_{i=1}^3 a_i \exp(-t/\tau_i) + a_4 \quad (1)$$

led to the results compiled in Table 1. These data seem to be in line with results obtained for the $P680^{\bullet+}$ reduction kinetics by measurements of 830 nm absorption changes in PSII preparations [32,33]. A closer inspection, however, reveals marked

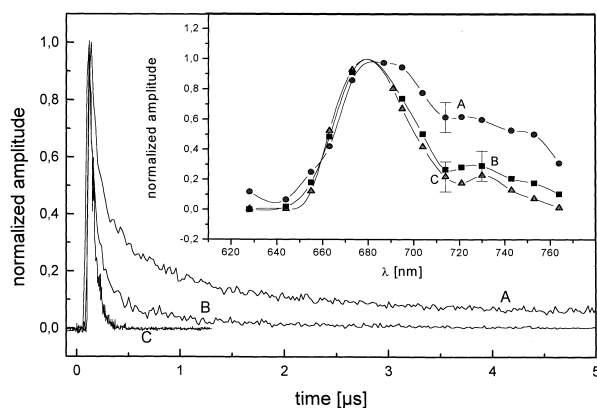


Fig. 2. Normalized time course of DF and amplitude at 100 ns after the flash as a function of wavelength (insert) in isolated thylakoids (curves labeled A), D1/D2/Cytb-559 (curves labeled C) preparations and solubilized LHCII (curves labeled B) from spinach. The experiments were performed at a Chl concentration of 10 $\mu\text{g/ml}$ (thylakoids) and 5 $\mu\text{g/mol}$ (LHCII and D1/D2/Cytb-559 preparations). The spectra in the insert are normalized to the peak maximum. Other experimental details as described in Section 2.

differences, especially with respect to the oscillation of the detectable initial amplitudes, DF (100 ns), and of the middle component. The slight oscillation of DF (100 ns) indicates that the redox state S_i of the WOC slightly affects either the quantum yield of $^1\text{P680}^*$ formation by $\text{P680}^{*+}\text{Q}_\text{A}^-$ recombination or the probability of radiative emission. Within the framework of the exciton radical pair model, these two parameters are most likely interrelated quantities as outlined in [34]. Therefore, an unambiguous answer cannot be given. It seems that the slight oscillation can be best explained by the idea that the redox state S_i of the WOC modulates the non-radiative decay rate in the PSII reaction center. The probability for this pathway is somewhat higher in S_2 and S_3 compared with that of S_0 and S_1 . The underlying mechanism of this effect remains to be elucidated.

A determination of the quantum yield of DF requires a suitable calibration method. D1/D2/Cytb-559 preparations which are able to generate the radical pair $\text{P680}^{*+}\text{Pheo}^{*-}$ but cannot perform the subsequent stabilization of this state because they are deprived of Q_A [35] are useful as standard for two reasons: (i) single photon counting (SPC) measurements which provide a complementary method for monitoring of DF by using very weak actinic flashes revealed that in this sample type the $\text{P680}^{*+}\text{Pheo}^{*-}$ state recombines in the ns time domain with high efficiency to $^1\text{P680}^*$ (two decay components with $\tau=20$ ns and 50 ns and quantum yield of 19% and 25%, respectively, were reported [36]) and (ii) the extent of 830 nm absorption changes induced by excitation with saturating laser flashes indicates that almost all D1/D2/Cytb-559 complexes form the radical pair $\text{P680}^{*+}\text{Pheo}^{*-}$ [21]. Typical traces of the normalized decay of DF in thylakoids and D1/D2/Cytb-559 preparations are depicted in Fig. 2. The DF decay of D1/D2/Cytb-559 satisfies a monoexponential decay with $\tau=52$ ns. This value perfectly corresponds with that of the slow component in [36], whereas the 20 ns component escapes detection in the time domain of 100 ns–5 μs . Therefore, the 25% value of [36] is used as a reference for the determination of the DF quantum yield in the thylakoids. The traces of Fig. 2 are normalized curves. The ratio of the absolute value of the

DF amplitudes extrapolated to $t=0$ after the actinic flash (symbolized by I_0) in D1/D2/Cytb-559 preparations and thylakoids was found to be about 2×10^4 when normalized to the same Chl concentration, i.e. the absorbed light of the actinic flash (I_abs) is virtually the same of both sample types. The quantum yield of DF is given by

$$\Phi_\text{DF} = \frac{I_0}{I_\text{abs}} * \sum_{i=1}^n a_i \tau_i \quad (2)$$

When using the experimental data of this study and the (Φ_DF value of 0.25 for D1/D2/Cytb-559 preparations in [36]a, a value of ($\Phi_\text{DF}(\text{thyl})=3 \times 10^{-5}$ is obtained for the DF emission from thylakoids up to 5 μs after the actinic flash. This value is lower than previously reported data [2–4,37]. It has to be emphasized that our calculation is restricted to a limited time range and therefore does not account for contributions owing to slow components. Regardless of the exact values, our results clearly show that the forward reactions in PSII with intact WOC are very efficient and dissipation via DF emission up to the levels of $\text{Y}_\text{Z}\text{P680}^{*+}\text{Q}_\text{A}^{*-}$ and $\text{Y}_\text{Z}^\text{ox}\text{P680Q}_\text{A}^{*-}$ is rather low.

The DF emission from PSII preparations reported so far was discussed within the framework of excited singlet state formation through reversal of the forward electron transfer processes. However, neither 3-(3,4-dichlorophenyl)-1,1-dimethylurea nor NH_2OH addition eliminated the DF emission in the ns time domain (data not shown) although both treatments are known to prevent the fast P680^{*+} reduction under repetitive flash excitation [38]. Therefore, the question arises as to whether excitation also leads to formation of trapped states in the antenna system. To the best of our knowledge, no DF data are reported for isolated antenna complexes. However, measurements of thermoluminescence suggest that illumination of antenna complexes gives rise to formation of traps that can regenerate excited singlet states [39]. The normalized DF traces of solubilized LHCII preparations are also shown in Fig. 2. A fit of the decay leads to biexponential kinetics with τ values of about 65 ns ($a_1=0.97$) and 800 ns ($a_2=0.03$). These kinetics are at least one order of magnitude slower than the dominating longest lifetime of prompt fluorescence (4.3 ns, see [40,41]) and clearly indicate that isolated LHCII exhibits DF emission. When using Eq. 2 and $\Phi_\text{DF}(\text{D1/D2/Cytb-559})=0.25$, a DF quantum yield of $4\text{--}5 \times 10^{-4}$ is obtained which exceeds that of thylakoids by more than a factor of 10. This finding is an independent line of evidence for the very efficient trapping of excitation energy by the reaction center of PSII.

The traces depicted in Figs. 1 and 2 were monitored at wavelength close to the emission peak of prompt fluorescence of Chl bound to proteins. In order to show that the DF really originates from Chl, the emission spectra of the three samples were measured. The results obtained are compiled in the insert of Fig. 2. The spectra normalized to the peak amplitude are characterized by a pronounced peak around 680 nm and thus indicative for radiative emission from the lowest excited singlet state of Chl *a*. Differences exist in the region of 720–740 nm where a markedly larger band is observed for thylakoids. Several effects could be responsible for this phenomenon. The possibility on an emission from ‘far red’ Chl *a* forms of PSI can be excluded [42]. Alternatively, the spectrum of thylakoids could be affected by reabsorption so that in the normalized

spectrum the 720–740 nm band would be more pronounced. Check experiments with samples of a different Chl concentration (data not shown) indicate that effects owing to reabsorption are of relevance but do not fully account for the enhanced ‘far red’ emission. The remaining part of the 720–740 nm band is ascribed to a vibronic satellite that is somewhat stronger in thylakoids than in D1/D2/Cytb-559 preparation and isolated LHCII. Extent and origin of this difference between thylakoids and the other two samples are not known and remain to be clarified in future studies.

4. Conclusion

The present study is the first report on DF emitted from thylakoids, D1/D2/Cytb-559 preparations and solubilized LHCII from spinach in the ns time domain after excitation with strong actinic flashes. The results obtained indicate that in thylakoids, the probability is very low for excited singlet state formation from a reversal of the forward reactions leading to radical pairs $Y_ZP680^{+}Q_A^{\bullet-}$ and $Y_Z^{ox}P680Q_A^{\bullet-}$. Excitation of solubilized LHCII complexes leads to weakly trapped states that recombine $^1Chl^*$ in the time range of 50 ns–1 μ s. This feature probably reflects the formation of a small fraction of radical pairs ($Chl^{\bullet+}Chl^{\bullet-}$) in antenna complexes. The implications of these findings for the photosynthetic apparatus and its sensitivity to light stress will be analyzed in forthcoming studies.

It has to be emphasized that in some cases, time-resolved monitoring of delayed light emission can also be achieved by using SPC techniques. However, the new method described here has essentially two advantages: (i) sensitive detection of DF in a wider time range and (ii) using of saturating actinic flashes to populate definite redox states in PSII, especially of the S_i states in the WOC.

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