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MULTIFREQUENCY CROSS-CORRELATION PHASE FLUOROMETRY OF CHLOROPHYLL *a* FLUORESCENCE IN THYLAKOID AND PSII-ENRICHED MEMBRANES

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Abstract—We present here a comparative study on the decay of chlorophyll (Chl) *a* fluorescence yield in thylakoid membranes and photosystem II (PSII)-enriched samples, measured with multifrequency cross-correlation phase fluorometry. These measurements confirm the general conclusions of Van Mieghem *et al.* (*Biochim. Biophys. Acta* **1100**, 198–206, 1992), obtained with a flash method, on the effects of reduction of the primary quinone acceptor (Q_A) on Chl *a* fluorescence yield of PSII. Different states of the reaction centers of PSII were produced by: (1) pretreatment with sodium dithionite and methyl viologen followed by laser illumination: the doubly reduced Q_A (Q_AH_2) centers; (2) with laser illumination or pretreatment with diuron: Q_A^- centers; and (3) the addition of micromolar concentration of dichlorobenzoquinone (DCBQ): oxidized Q_A centers. The data were analyzed with Lorentzian distribution as well as with multiexponential fluorescence decay functions. The analysis with Lorentzian distribution function showed that upon formation of Q_A^-, the major lifetime distribution peak shifted to longer lifetimes: from 0.25 ns to 1.66 ns (pea thylakoid membranes) and from 0.24 ns to 1.31 ns (core PSII). However, when Q_AH_2 was formed, the lifetime distribution peaks shifted back to shorter lifetimes (0.57–0.77 ns) both in thylakoids and PSII membranes. Multiexponential analysis showed three lifetime components: fast (40–400 ps), middle (300–1500 ps) and slow (5–25 ns). When Q_A^- was formed in PSII centers, the amplitude of the fast component decreased, but both the amplitude and the lifetime of the middle component increased severalfold. However, when Q_A was doubly reduced, the amplitude of the fast component increased and the amplitude of the middle component decreased; in addition, the lifetime of the slow component increased. All of the above results are consistent with the conclusions that PSII charge separation is decreased when Q_A^- is formed and increased when doubly reduced Q_A is formed.

INTRODUCTION

In chloroplasts, light energy is captured by antenna pigments that are contained in chlorophyll (Chl)† *a/b* or Chl *a*–protein complexes. The excitation energy is transferred through a series of ultrafast energy transfer steps in tens of picoseconds before it reaches the reaction centers for photochemical charge separation. Most of Chl *a* fluorescence at room temperature originates in photosystem II (PSII). The yield of this PSII fluorescence is dependent on the redox state of the reaction center. When the electron acceptor Q_A of PSII is in the oxidized state, the Chl *a* fluorescence yield is low (F_o). However, when the Q_A is reduced, the Chl *a* fluorescence yield is high (F_{max}). Along with the increase in fluorescence yield a parallel increase in the fluorescence lifetime is expected, which gives direct access to measurement of the excitation energy transfer and charge-separation processes. For reviews on Chl *a* fluorescence, see Govindjee *et al.*,[1] Holzwarth,[2,3] Karukstis[4] and Krause and Weis.[5]

Measurements of the lifetime of Chl *a* fluorescence after picosecond excitation of photosynthetic organisms, containing light-harvesting Chl *a/b* protein complexes, have revealed multiexponential decay kinetics.[2,3,6,7] The overall fluorescence decay can usually be statistically defined by four major components: ultrafast (10–20 ps), fast (40–400 ps), middle (300–1500 ps) and slow (1.2–35 ns), each of which may be composed of several subcomponents. These undergo complex changes in both their lifetimes and yields upon closure of PSII reaction centers to photochemistry.[8–14] The complexity of interpretation is due to the existence of energy transfer events within the pigment–protein complexes, as well as among the various complexes that are of heterogeneous character and the inclusion of the influence of charge separation events on the lifetime of Chl *a* fluorescence yield.[3]

The origins of each of these components are still not fully understood. It has been shown, from time-resolved emission spectra[15,16] and from PSI and PSII mutant studies,[17] that a part of the rapid decay arises from PSI (30 ps; 100–150 ps) and another (100–350 ps) from PSII. Klimov *et al.*[18] proposed that a slow component (1.3–2.5 ns) originates from PSII radical pair recombination between the oxidized reaction center Chl *a* of PSII, $P680^+$, and reduced primary acceptor (pheophytin) of PSII, $Pheo^-$, an idea that may not be valid for intact PSII,[3] but may be applicable to reaction center II preparations for the 2–35 ns component (see *e.g.* Govindjee *et al.*[14]).

The idea that the complexity of the kinetic components of fluorescence decay could be interpreted in terms of the existence of two types of PSII centers PSIIα and PSIIβ (see *e.g.* Melis and Homann,[19] and as done by Holzwarth[3]) was first suggested by Butler *et al.*[20] (also see other references[21,22]).

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†*Abbreviations:* Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU (diuron), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethyl-*p*-benzoquinone; FWHM, full width at half maximum; MES, 2-(*N*-morpholino)ethanesulfonic acid; P680, primary electron donor of reaction center II; Pheo, pheophytin; PSII, photosystem II; Q_A, bound plastoquinone, a one electron acceptor of photosystem II.

Based on time-resolved emission and excitation spectral analyses performed under conditions when reaction centers are open (F_o, Q_A is oxidized) and when they are closed (F_{max}, Q_A is reduced), Holzwarth *et al.*[23] had proposed that an 80 ps component arises from PSI, a 180 ps component from open PSIIα centers, the original "middle" component (500 ps) from open, and slow (1.2 ns) from closed PSIIβ centers, while the long-lived (2.2 ns) component is emitted by closed PSIIα centers. However, Holzwarth[3] later suggested that the 1.2 ns component is from closed PSIIα and the 2.2 ns from PSII closed PSIIβ.

Using a simpler system, *i.e.* PSII particles and low intensity of excitation, Schatz and coworkers[12,24] observed that upon closure of reaction centers (Q_A^--closed), lifetimes of fluorescence changed from about 80 ps and 520 ps to 220 ps and 1–3 ns. Schatz *et al.*[25] suggested that Q_A^- controls primary charge separation; with Q_A^- present, the charge separation was slowed/decreased, as confirmed by Trissl *et al.*[26]: the 520 ps time was related to the time of electron transfer from the reduced Pheo to Q_A.

Discrete component analysis of the fluorescence assumes that all the radiating fluorophores decay with a well-defined set of lifetimes. However, in the case of heterogeneous systems containing proteins, membranes, *etc.*, in which the electronic environments of the emitting molecules are far from being unique and can change during the excited state lifetime, such an approach has been questioned.[27] The simulation study of Alcala *et al.*,[28] for protein fluorescence, showed that the discrete component analysis with one or two exponentials, when used to study distributions of lifetimes, was very sensitive to the number and range of frequencies at which the data are collected. In general a two exponential fit to a symmetric distribution yielded a nonsymmetric result. The result of the fit was symmetric only with very particular sets of frequencies whose values depended on the distribution shape. To distinguish among the different factors involved in the decay is impossible due to the limited resolvability of the data in lifetime components provided by current instrumentation. Thus, the observed signal may, alternatively, be easily composed of a superposition of heterogeneous decays comprising individual lifetime values that are close to one another.[29] As a result, the assignment of one or more exponentials to describe the overall decay process can hide the true physical origin of lifetime heterogeneity.

Photosystem II is known to be a Chl-protein-containing heterogeneous membrane system.[30] It is well established that protein structural fluctuations can occur in the nanosecond–picosecond time scale.[31–33] The concept of distribution of lifetime values has been introduced in fluorescence[34] and has been successful in lifetime analysis of Chl *a* fluorescence[14] of reaction center II preparations[35] that lack Q_A.

We present here an analysis of fluorescence decay data in thylakoid membranes and in two different PSII-enriched membranes, using multifrequency phase fluorometry. A Lorentzian analysis is made because the fluorescence decay may be a superposition of many similar exponential decays, as noted above. In particular, we have used PSII samples similar to those used by Van Mieghem *et al.*[36] in order to test their conclusions regarding the influence of the redox state of Q_A on the charge transfer by the multifrequency cross-correlation phase fluorometry. These data were analyzed for both open and closed PSII centers in terms of distributions of lifetimes based on the principles outlined by Alcala *et al.*[27,29,37] (also see Govindjee *et al.*[14]). Analysis of the data with both multiexponential and Lorentzian distribution functions showed that upon closure of reaction centers (Q_A^--closed centers), the lifetime peak of the major Lorentzian distribution shifted to longer lifetimes: from 0.25 ns to 1.66 ns in pea thylakoid membranes; and from 0.24 ns to 1.31 ns in PSII membranes. This change in fluorescence properties of PSII may be caused by a transmembrane electric field and the charge of Q_A^- (see Keuper and Sauer[13] and Holzwarth[3]). However, in samples in which Q_A was doubly reduced, the lifetime distribution (peak at 0.57 ns for spinach thylakoids and at 0.77 ns for PSII) had shorter lifetimes compared to that of Q_A^--closed centers and longer lifetimes compared to that of the open centers (Q_A centers). In this case, the effect of the charge disappears as doubly reduced Q_A may become quinol, as suggested by Van Mieghem *et al.*[36] Thus, our data on our PSII samples, measured with an independent method, complement the published data[36] and support the earlier conclusions.

MATERIALS AND METHODS

Thylakoid and PSII membranes were prepared from appressed membrane fragments of chloroplasts from *Pisum sativum* (peas), as described by others (see K and M preparation in Dunahay *et al.*[38]). A PSII sample, prepared by the method of Ghanotakis *et al.*,[39] labeled as "core PSII," was also used in this study. In addition, thylakoids and PSII-enriched membranes (Berthold *et al.*[40]) were prepared from spinach (*Spinacia oleracea*). The Chl concentration was determined using extinction coefficients published by Ziegler and Egle[41] for absorbance at 664 nm and 647 nm.

Samples were suspended in a reaction medium containing 0.4 *M* sorbitol, 5 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH (pH 6.5), 20 m*M* KCl, 2 m*M* $MgCl_2$ and 1 μM nigericin when fluorescence was measured. The Chl concentration was 5 μM. The F_o (open centers) condition was obtained by the addition of 15 μM 2,6-dichloro-*p*-benzoquinone (DCBQ) and the F_{max} by 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Q_A^--closed centers). In addition, in other samples Q_A was doubly reduced by adding 15 m*M* freshly prepared sodium dithionite and 150 μM methyl viologen and leaving the sample to incubate in the dark for about 2 h before use (modified after Van Mieghem *et al.*[36]; this is a much more drastic treatment than used for chemical reduction of Pheo; see *e.g.* Wasielewski *et al.*[42]). The double reduction of Q_A was tested as in Van Mieghem *et al.*[43] by Govindjee (data not shown). The samples were kept in tightly covered cuvettes during measurements.

To study the time-resolved fluorescence emission, a multifrequency cross-correlation phase fluorimeter was used. The light source consisted of a Coherent Antares 76-S neodymium yttrium–aluminum–garnet (Nd-YAG) laser, mode locked at 76 MHz. The picosecond optical pulse train generated by this system synchronously pumped a cavity-dumped, model 701-3 rhodamine 6G dye laser (Coherent). The repetition rate of the Coherent model 7200 cavity dumper was set at 2 MHz. The sample was excited under "magic angle" conditions at 610 nm with an attenuated, collimated 1 mW beam. The emission was observed at 680 nm through a UV/vis F/3.5 monochromator (Instruments SA model H10) equipped with a concave holographic grating with 1200 grooves/mm. Bandwidths of 8 nm full width at half maximum (FWHM) were used throughout the experiments. Both reference and sample detectors consisted of highly sensitive, low-dark-noise Hamamatsu R-928 photomultipliers operated at room temperature. Radiofrequency signals were obtained from a Marconi model 2022A signal generator and subsequently amplified by an Electronic Navigation Instruments model 603L RF power amplifier. The cross-correlation signal was set at 40 Hz (see details in earlier publications[14,28,44]).

When a fluorescence system is excited by a sinusoidally modulated light intensity at an angular frequency ω, we have:

$$E(t) = E_0(1 + M_e \sin \omega t) \quad (1)$$

where E_0 is the average intensity and M_e is the modulation of the excitation. The fluorescence response of the system can be written in the form

$$F(t) = F_0[1 + M_f \sin(\omega t - \phi)] \quad (2)$$

where F_0 and M_f are the average fluorescence and its modulation. The fluorescence is phase shifted with respect to the excitation by a value ϕ and demodulated such that the ratio $M = M_f/M_e < 1$. At a given modulation frequency, the measurable quantities ϕ and M are related to the physical parameters of fluorescence population by the following equations[27]:

$$\phi = \tan^{-1} S(\omega)/G(\omega) \quad (3)$$

$$M = [S^2(\omega) + G^2(\omega)]^{1/2} \quad (4)$$

where

$$S(\omega) = \int_0^\infty I_F(t) \sin \omega t \, dt/N \quad (5)$$

$$G(\omega) = \int_0^\infty I_F(t) \cos \omega t \, dt/N. \quad (6)$$

The function I_F contains information on the distribution of components in the time domain. $S(\omega)$ and $G(\omega)$ are the sine and cosine Fourier transformations of I_F and N, a normalization factor.[27] Phase and modulation data were generated by using sets of components with amplitudes determined according to a given distribution function. Analysis of the data with multiexponential function assumed that the light emission by a fluorescence sample upon delta-function excitation can be described as a superposition of discrete exponential decays (see earlier publications[14,28,44]).

RESULTS

Lifetime of Chl a fluorescence of PSII when Q_A is reduced

We measured lifetimes of fluorescence at the two extremes: (F_0) when all Q_A was oxidized and (F_{max}) when all Q_A was reduced because the kinetics of decay are simplified and the analyses are unaffected by the organization of the PSII units (*i.e.* they are independent of whether the matrix [*i.e.* "lake"] or separate package [*i.e.* "isolated puddles"] model is appropriate). As noted earlier, the samples were excited at 610 nm and the Chl *a* fluorescence was measured at 680 nm. Figure 1 shows the phase shift, Φ, and relative modulation, M, as a function of frequency in MHz in open and Q_A^--closed PSII centers in pea thylakoid membranes (Fig. 1A) and pea core PSII (Fig. 1B). The open (F_0) condition[45] was ascertained by the addition of 15 μM DCBQ, whereas the Q_A -closed condition was obtained by the addition of 5 μM DCMU in both pea thylakoid membranes and PSII samples. Closure of PSII centers causes large changes in both demodulation M and phase shift Φ (compare data with open and closed symbols). The results of multiexponential model fits are shown in Table 1. In order to fit the data, a minimum of three exponential components are necessary at both F_0 (open centers) and F_{max} (Q_A^--closed centers) as judged by the low residuals (Fig. 2) and the low χ^2 values. A single or double exponential fit is not sufficient to describe the data (data not shown). These results are qualitatively, but not quantitatively, in agreement with those published earlier.[13,16] Analysis with four decay components led to only a slight lowering of the χ^2 and produced an additional component with an insignificant amplitude.

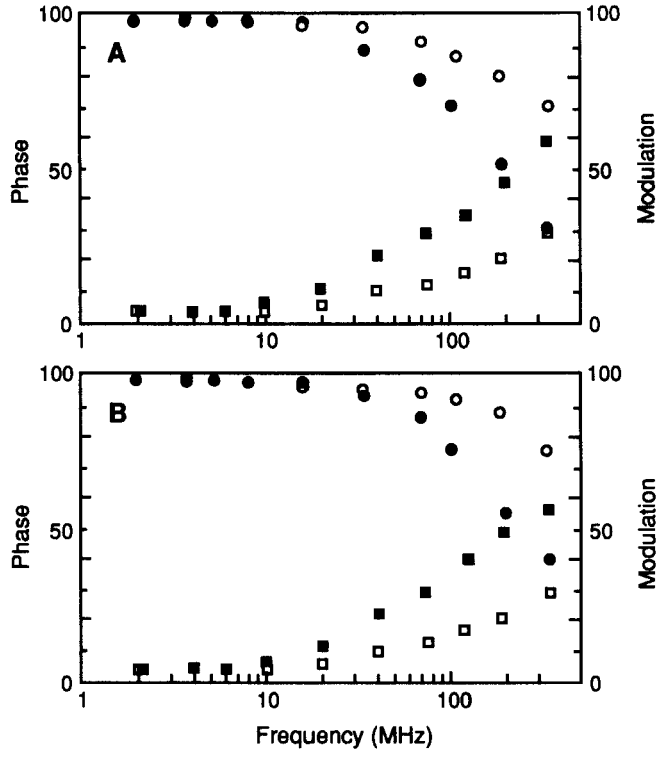

Figure 1. Phase (in degrees; squares) and modulation (full scale, 100%; circles) as a function of frequency for the Chl *a* fluorescence decay in pea thylakoid membranes (A) and core PSII (B) in the presence of 5 μM DCMU (closed symbols) (Q_A^--closed PSII centers) and 15 μM DCBQ (open symbols) (open PSII centers). Samples were suspended in a reaction medium containing 0.4 *M* sorbitol, 5 m*M* MES-KOH (pH 6.5), 20 m*M* KCl, 2 m*M* $MgCl_2$ and 1 μM nigericin. The Chl concentration was 5 μM.

When Q_A remained mostly oxidized in the presence of DCBQ, three resolved lifetime components in the open PSII centers in the core PSII were approximately 40 ps (32%, fractional intensity), 480 ps (65%) and 8.5 ns (negligibly small, 3%), and those in pea thylakoid membranes were approximately 40 ps (30%), 600 ps (64%) and 5.9 ns (again, very small, 6%). Upon closure of the PSII centers (Q_A -closed), the three lifetime components were approximately 50 ps (only 8%), ~1.3 ns (85%) and ~5.6 ns (only 7%) in core PSII; in thylakoid membranes these components were approximately 140 ps (11%), 1.6 ns (78%) and 5.1 ns (11%). Thus closure of the reaction centers, *i.e.* formation of Q_A^-, leads to two- to three-fold increase of the lifetime of the middle component, but the fractional intensity increases only by a factor of 1.2–1.3. The slowest component is of no significant consequence in view of its very low contribution. The major observations here are the three- to four-fold decrease in the fractional intensity of the fast component as Q_A is reduced to Q_A in both PSII core and thylakoid membranes, and the two- to three-fold increase in the lifetime of the middle component; in fact the middle component apparently becomes a new slow component. These observations not only confirm the conclusions of Van Mieghem *et al.*[36] but provide a more reliable lifetime value of the fast component in view of our better time resolution. Untreated control samples give results similar to that of Q_A^--closed centers, as the high intensity

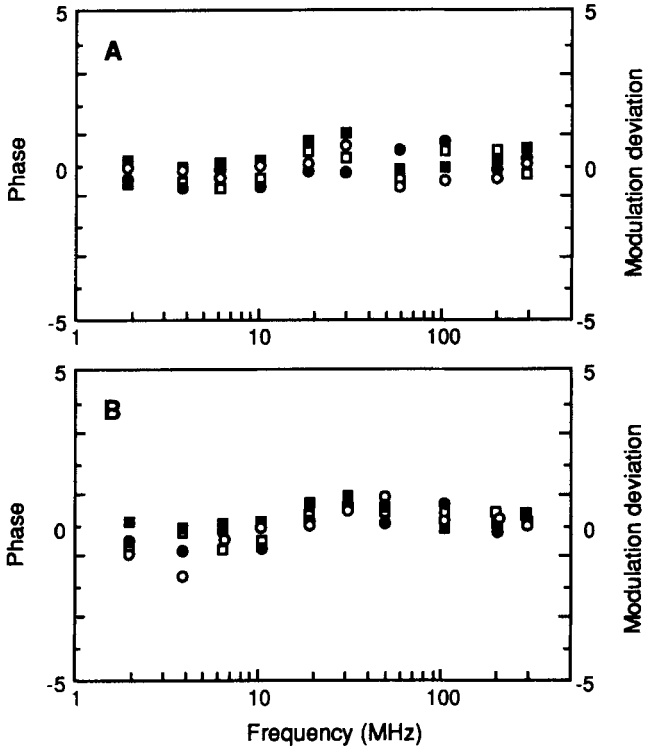

Figure 2. Phase (in degrees; squares) and modulation (with full scale as 100%; circles) deviations given as weighted residuals between the calculated and experimental data of Fig. 1 are shown in the middle of the plot with scale +5 to −5. (A): pea thylakoid membranes. (B): core PSII. All symbols are as described in the legend of Fig. 1. The nonlinear least-squares data analysis procedure was applied.

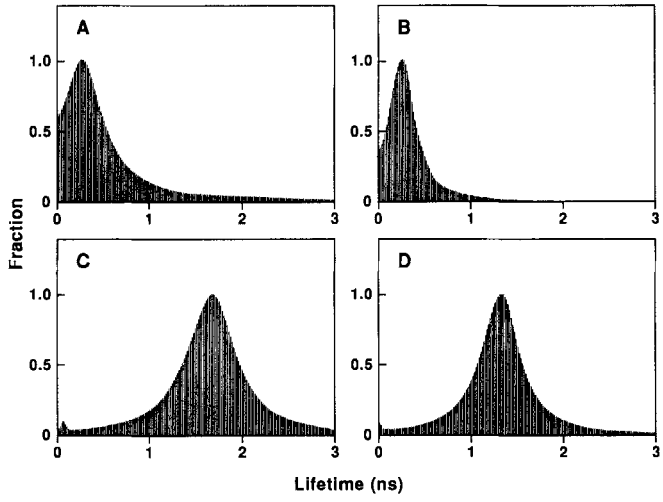

Figure 3. Lorentzian lifetime distributions for the Chl *a* fluorescence decay in pea thylakoid membranes (A and C) and core PSII (B and D) in the presence of 5 μM DCMU (C and D: Q_A^--closed PSII centers) and 15 μM DCBQ (A and B: open PSII centers). The open center data were fitted with a single Lorentzian function, and the Q_A^--closed center data were fitted with a double Lorentzian function. The other details are given in the legend of Fig. 1.

of light, used here, also reduces all Q_A to Q_A^- (data not shown).

Now, we show results with the alternate lifetime distribution method, the focus of this paper. Analysis with Lorentzian functions gives lifetime distributions shown in Fig. 3; the parameters for Lorentzian fit functions are presented in Table 2. In open PSII centers, *i.e.* with all Q_A in the oxidized state, a single Lorentzian lifetime distribution with a center at about 240 ps (PSII core; curve B) or 250 ps (thylakoid membranes; curve A) is obtained. This contrasts with the three exponential decay analysis. In closed PSII centers, *i.e.* with all Q_A in the reduced state, a double Lorentzian lifetime distribution with a dominant distribution peaking at 1.3 ns (PSII core; curve D) or 1.7 ns (thylakoid membranes; curve C) was observed; a picosecond peak has a negligibly small fractional intensity of 5% or 7%. A shift from a shorter lifetime distribution to a longer lifetime distribution occurred upon closure of PSII (Q_A^- closed), as expected.

PSII centers with double reduction of Q_A

It appears that DCBQ opens most of the PSII centers by oxidizing the primary quinone acceptor Q_A. On the other hand, pretreatment with benzyl or methyl viologen and sodium dithionite and light leads to double reduction of Q_A (see Van Mieghem *et al.*[36]). Figure 4 shows the phase shift, Φ, and relative modulation, M, as a function of frequency in MHz in Q_A^- and in doubly reduced Q_A PSII centers in spin-

Table 1. Lifetime (τ) and fractional intensity (f) obtained by a triple exponential fit to the fluorescence decay of thylakoid membranes (peas) and core PSII*

	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	f_1	f_2	f_3	χ^2
Open centers							
Core PSII	0.04	0.48	8.46	0.32	0.65	0.03	3.65
Thylakoid membranes	0.04	0.60	5.89	0.30	0.64	0.06	2.30
Q_A^--closed centers							
Core PSII	0.05	1.27	5.56	0.08	0.85	0.07	1.77
Thylakoid membranes	0.14	1.55	5.10	0.11	0.78	0.11	2.05

*The Q_A -closed PSII centers in the samples were obtained by adding 5 μM DCMU, and open PSII centers were obtained by adding 15 μM DCBQ. In this experiment, thylakoids were prepared from peas.

Table 2. Center (c), width (w) and fractional intensity (f) obtained by a Lorentzian distribution model for the fluorescence decay of thylakoid membranes and Core PSII*

	c_1 (ns)	c_2 (ns)	w_1 (ns)	w_2 (ns)	f_1	f_2	χ^2
Open centers							
Core PSII	0.24	—	0.34	—	1.00	—	4.72
Thylakoid membranes	0.25	—	0.59	—	1.00	—	3.43
Q_A^--closed centers							
Core PSII	0.01	1.31	0.05	0.95	0.05	0.95	1.91
Thylakoid membranes	0.37	1.66	0.05	0.93	0.07	0.93	1.46

*The Q_A^--closed PSII centers in the samples were obtained by adding 5 μM DCMU, and open PSII centers were obtained by adding 15 μM DCBQ.

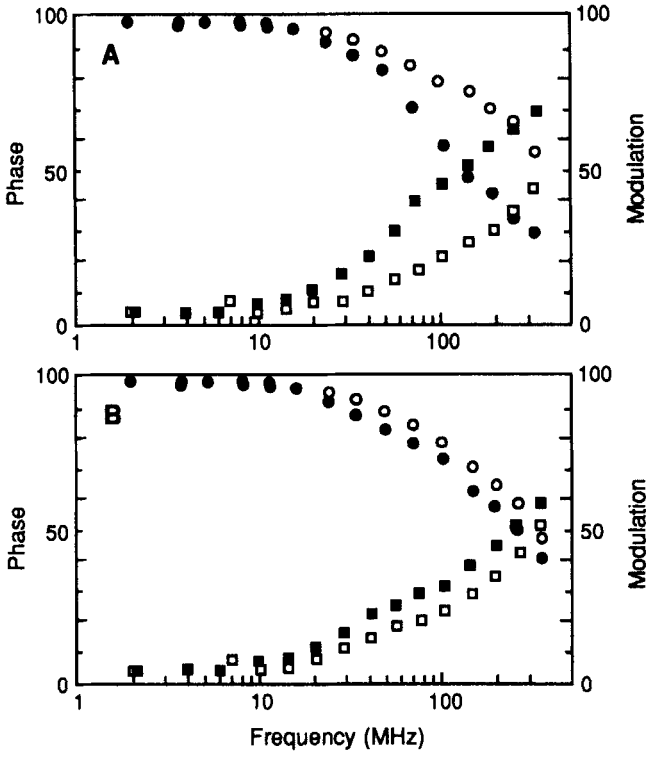

Figure 4. Phase (in degrees; squares) and modulation (full scale, 100%; circles) as a function of frequency for the Chl *a* fluorescence decay in spinach thylakoid membranes (A) and PSII membranes (B) of Q_A^--closed centers (closed symbols) and of doubly reduced Q_A centers (open symbols). See Materials and Methods. Illumination after treatment with dithionite and methyl viologen was done with a standard flashlight and subsequently with 1.5 W 532 nm laser light for 2 min. The Q_A^--closed centers were generated simply by illumination with the laser.

ach thylakoid membranes (Fig. 4A) and PSII membranes (Fig. 4B). Samples with doubly reduced Q_A have significantly different demodulation M and phase shift Φ from those with singly reduced Q_A.

Parameters obtained by a triple exponential fit for the above fluorescence data are presented in Table 3. In Q_A^--closed

Table 3. Lifetime (τ) and fractional intensity (f) obtained by a triple exponential fit to the fluorescence decay of thylakoid membranes (spinach) and PSII membranes*

	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	f_1	f_2	f_3	χ_2
Q_A^--closed centers							
PSII membranes	0.38	1.42	8.11	0.21	0.76	0.03	7.95
Thylakoid membranes	0.34	1.79	4.46	0.09	0.76	0.15	13.88
Doubly reduced Q_A							
PSII membranes	0.41	1.33	24.4	0.45	0.49	0.06	5.95
Thylakoid membranes	0.21	0.79	5.63	0.27	0.62	0.11	3.91

*Samples with doubly reduced Q_A were prepared in the presence of sodium dithionite and methyl viologen plus laser illumination, and Q_A^--closed PSII centers were obtained by simply illuminating samples with laser light. In this experiment, thylakoids were prepared from spinach.

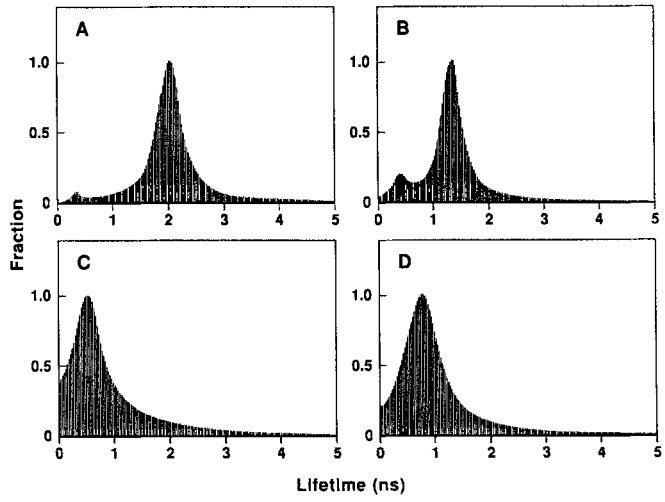

Figure 5. Lifetime distribution for Chl *a* fluorescence decay in spinach thylakoid membranes (A and C) and PSII membranes (B and D) in the presence of laser illumination for the Q_A^--closed centers (A, B) and sodium dithionite and methyl viologen plus laser light for doubly reduced Q_A (C, D); also see legend for Fig. 4 and Materials and Methods. Data were best fit with double and single Lorentzian functions, respectively.

PSII centers, the lifetime of the fast decay component is approximately 340 ps (9%, fractional intensity) in spinach thylakoid membranes and 380 ps (21%) in PSII membranes; the lifetime of the middle decay component is approx. 1.8 ns (76%; thylakoids) and 1.4 ns (76%; PSII membranes); and that of the slowest component is 4.5 ns (only 15%; thylakoids) or 8.1 ns (only 3%; PSII membranes). Upon double reduction of Q_A, the major changes are a two- to three-fold increase in the fraction of the fast component and a smaller decrease for the fraction of the middle component; in addition, in PSII membranes, there is also an increase in the lifetime of the slow component. These observations are quite consistent with those obtained by Van Mieghem *et al.*[36]

Figure 5 shows double Lorentzian function lifetime distributions of spinach thylakoid membranes (Fig. 5A,C) and PSII membranes (Fig. 5B,D); the fit parameters for Lorentzian functions are presented in Table 4. In Q_A^--closed centers, the majority of fractional intensity is localized at a Lorentzian

Table 4. Center (c), width (w) and fractional intensity (f) obtained by a double Lorentzian distribution model for the fluorescence decay of spinach thylakoid membranes and PSII membranes*

	c_1 (ns)	c_2 (ns)	w_1 (ns)	w_2 (ns)	f_1	f_2	χ^2
Q_A^--closed centers							
PSII membranes	0.37	1.37	0.05	0.36	0.16	0.84	8.51
Thylakoid membranes	0.34	2.00	0.05	0.54	0.07	0.93	14.56
Doubly reduced Q_A							
PSII membranes	0.77	—	0.76	—	1.00	—	1.31
Thylakoid membranes	0.57	—	0.70	—	1.00	—	3.48

*The samples with doubly reduced Q_A and those with reduced Q_A were obtained as described in the legend for Table 3. In this experiment, thylakoids were prepared from spinach.

function center at 2 ns (thylakoid membranes; Fig. 5A) or 1.37 ns (PSII-enriched membranes; Fig. 5B). In samples with doubly reduced Q_A, however, the distribution peak is shifted to shorter lifetimes. The main centers of the Lorentzian function fit for thylakoid membranes and for PSII are at 0.57 ns (Fig. 5C) and at 0.77 ns (Fig. 5D).

DISCUSSION

In this paper, we have presented our measurements on Chl *a* fluorescence lifetimes in the open (all oxidized Q_A), closed (all reduced Q_A) and those with doubly reduced Q_A PSII centers in thylakoid membranes and PSII-enriched membranes, using multifrequency phase fluorometry. The fluorescence decay data were analyzed in terms of the usual multiexponential fluorescence decay and the alternate Lorentzian distribution functions. Multiexponential analysis of Q_A-containing PSII centers showed three lifetime components: fast (40–140 ps), middle (400–600 ps) and slow (5–8 ns). Upon closure of the PSII centers (Q_A^- closed), both the lifetime and the amplitude of the middle component increased, whereas the amplitude of the fast component decreased. This is consistent with the concept[3] that with reduced Q_A, the probability of charge separation in PSII decreases. The analysis with Lorentzian distribution functions, the focus of this paper, showed that in Q_A^--closed centers, the lifetime peaks of the major Lorentzian distribution showed a dramatic shift from 0.23–0.25 ns to 1.3–1.6 ns, whereas, in samples with doubly reduced Q_A, the lifetime peaks were shifted back to much lower lifetimes (0.57–0.77 ns) than those in samples with Q_A^--closed centers.

Our results on thylakoids are in qualitative agreement with the reports by Haehnel *et al.*[8] and by Keuper and Sauer.[13] The increase in the fractional intensity of the slow component, however, was smaller in the current experiment. Here, we have confirmed that upon closure of the PSII centers (Q_A^- closed), a two- to three-fold increase in the lifetime and a slight increase in fractional intensity of the middle component, a relatively small or no change in lifetime and a three- to four-fold decrease in fractional intensity of the fast component and a small increase of the slow component occurs in PSII core and PSII membranes as well as in thylakoids. Our results are also in line with the observation that the yield of the slow component is close to zero (0.04) (*cf.* Haehnel *et al.*[8]) in the presence of DCBQ, which seems to keep most PSII centers open. The fast component showed a decrease in the yield from a high value (0.30) to a very low value (0.10) when PSII centers were closed. This provides strong evidence that the fast component is controlled by the process of energy conversion in the open PSII reaction centers.[3]

Schatz *et al.*[12] showed that charge separation in PSII reaction centers is trap limited. This is consistent with a linear relationship between the total charge separation time and the antenna size (see Pearlstein[46]). In open PSII centers the half-time of electron transfer from $Pheo^-$ to Q_A is 300–500 ps.[12,47] In closed centers the long-lived (larger than 2 ns) lifetime components for the radical pairs contained less than 10% of total fluorescence. The increase in Chl *a* fluorescence yield was considered to be caused by a lengthening of the excited state lifetime due to a decreased yield of charge separation. In contrast, Mauzerall[48] concluded that the long-lived (about 2 ns) fluorescence from closed PSII centers is recombination luminescence as proposed by Klimov *et al.*[18] However, recent experimental results do not support the hypothesis of recombination luminescence in Q_A-containing PSII.[5] Such recombinational luminescence, however, occurs[3,14] in PSII reaction centers devoid of Q_A.

In PSII samples that contain doubly reduced Q_A, lifetime distribution is shifted to shorter lifetimes from that in samples that contain reduced Q_A (Van Mieghem *et al.*[36]; this paper) but with somewhat longer lifetimes than those with open centers. This is explained by an increased probability of charge separation and by an increased probability of charge recombination. The electrostatic effect of Q_A^- on the $P680^+$ $Pheo^-$ radical pair is lost and these centers would resemble the open centers with short fluorescence lifetime.

The fluorescence decay is customarily resolved in terms of exponential components, and the values of the decay rates and preexponential factors of each component are associated with a particular conformation and with the relative population of each conformation. However, the preexponential factors cannot be related to the fraction of molecules in each conformation. The fluorescence lifetime distribution is determined by the multitude of conformational substates in a protein and by the dynamics of the protein (Gratton *et al.*[49]). The lifetime distribution method, as used here, provides a good approach for the study of conformational substates and of the energetics of such substates. In the limiting case of the frozen protein (negligible dynamics), one may consider that the fluorescence is determined by a set of exponentials of which the lifetimes and amplitudes are characteristic of the set of environments of the excited residues in the protein. However, as the dynamic nature of the protein is allowed to play its role, the excited chlorophylls become exposed to electronic environments, the nature of which vary with time. Here in this study, the changes in the lifetime distribution of Chl *a* fluorescence decay in Q_A^--closed centers and centers with doubly reduced Q_A have provided a newer and alternate view on the changes that occur when PSII reaction centers are closed.

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