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 (QA) 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 QA (QA(H2)) centers; (2) with laser illumination or pretreatment with diuron: QA− centers; and (3) the addition of micromolar concentration of dichlorobenzoquinone (DCBQ): oxidized QA centers. The data were analyzed with Lorentzian distribution as well as with multieponential fluorescence decay functions. The analysis with Lorentzian distribution function showed that upon formation of QA−, 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 QA(H2) was formed, the lifetime distribution peaks shifted back to shorter lifetimes (0.57–0.77 ns) both in thylakoids and PSII membranes. Multieponential analysis showed three lifetime components: fast (40–400 ps), middle (300–1500 ps) and slow (5–25 ns). When QA− 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 QA(H2) 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 PSI1 charge separation is decreased when QA− is formed and increased when doubly reduced QA(H2) 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 QA of PSII is in the oxidized state, the Chl a fluorescence yield is low (F0). However, when the QA is reduced, the Chl a fluorescence yield is high (Fmax). 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,2,7 Karukstis3 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 multieponential 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.5,6 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.5

The origins of each of these components are still not fully understood. It has been shown, from time-resolved emission spectra15,16 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,2 but may be applicable to reaction center II preparations for the 2–35 ns component (see e.g. Govindjee et al.19).

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 PSIIa and PSIIb (see e.g. Mclis and Homan,19 as done by Holzwarth20) was first suggested by Butler et al.20 (also see other references21,22).
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. reported that an 80 ps component arises from PSI, a 180 ps component from open PSI10 centers, the original “middle” component (500 ps) from open, and slow (1.2 ns) from closed PSI1/2 centers, while the long-lived (2.2 ns) component is emitted by closed PSI10 centers. However, Holzwarth later suggested that the 1.2 ns component is from closed PSI10 and the 2.2 ns from PSI closed PSI2.

Using a simpler system, i.e. PSI1 particles and low intensity of excitation, Schatz and coworkers 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. suggested that Q$_A$ controls primary charge separation; with Q$_A$ present, the charge separation was slowed/decreased, as confirmed by Trissl et al., 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. The simulation study of Alcala et al. 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. 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. It is well established that protein structural fluctuations can occur in the nanosecond-picosecond time scale. The concept of distribution of lifetime values has been introduced in fluorescence and has been successful in lifetime analysis of Chl a fluorescence of reaction center II preparations that lack Q$_A$.

We present here an analysis of fluorescence decay data in thylakoid membranes and in two different PSI1-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 PSI1 samples similar to those used by Van Mieghem et al. 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 PSI1 centers in terms of distributions of lifetimes based on the principles outlined by Alcala et al. (also see Govindjee et al.). Analysis of the data with both multieponential 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 PSI1 membranes. This change in fluorescence properties of PSI1 may be caused by a transmembrane electric field and the charge of Q$_A$ (see Keuper and Sauer and Holzwarth). 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 PSI1) 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. Thus, our data on our PSI1 samples, measured with an independent method, complement the published data and support the earlier conclusions.

**MATERIALS AND METHODS**

Thylakoid and PSI1 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.). A PSI1 sample, prepared by the method of Ghanotakis et al., labeled as “core PSI1,” was also used in this study. In addition, thylakoids and PSI1-enriched membranes (Berthold et al.) were prepared from spinach (*Spinacia oleracea*). The Chl concentration was determined using extinction coefficients published by Ziegler and Egel (for absorbance at 664 nm and 647 nm).

Samples were suspended in a reaction medium containing 0.4 M sorbitol, 5 mM 2-(N-morpholinophenethyl)sulfonic acid (MES)-KOH (pH 6.5), 20 mM KCl, 2 mM MgCl$_2$, and 1 mM 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) –closed centers). In addition, in other samples Q$_A$ was doubly reduced by adding 15 mM 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.); this is a much more drastic treatment than used for chemical reduction of Pheo; see e.g. Wricke et al.). The double reduction of Q$_A$ was tested as in Van Mieghem et al. 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 dumps, 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).
When a fluorescence system is excited by a sinusoidally modulated light intensity at an angular frequency $\omega$, we have:

$$E(t) = E_0(1 + M_0 \sin \omega t)$$

where $E_0$ is the average intensity and $M_0$ is the modulation of the excitation. The fluorescence response of the system can be written in the form

$$F(t) = F_0[1 + M_0 \sin(\omega t - \phi)]$$

where $F_0$ and $M_0$ are the average fluorescence and its modulation. The frequency is phase shifted with respect to the excitation by a value $\phi$ and demodulated such that the ratio $M = M_0/M_e < 1$. At a given modulation frequency, the measurable quantities $\phi$ and $M$ are related to the physical parameters of fluorescence population by the following equations:

$$\phi = \tan^{-1} \frac{S(\omega)}{G(\omega)}$$

$$M = \left[ S'(\omega) + G'(\omega) \right]^{1/2}$$

where

$$S(\omega) = \int_0^\infty I_1(t) \sin \omega t \, dt / N$$

$$G(\omega) = \int_0^\infty I_1(t) \cos \omega t \, dt / N.$$  

The function $I_1$ contains information on the distribution of components in the time domain. $S(\omega)$ and $G(\omega)$ are the sine and cosine Fourier transformations of $I_1$ and $N$, a normalization factor. 

## RESULTS

### Lifetime of Chl a Fluorescence of PSII When QA is Reduced

We measured lifetimes of fluorescence at the two extremes: $(F_0)$ when all QA was oxidized and $(F_{max})$ when all QA 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, $\phi$, and relative modulation, $M$, as a function of frequency in MHz in open and closed PSII centers in pea thylakoid membranes (Fig. 1A) and pea core PSII (Fig. 1B). The open $(F_0)$ condition was ascertained by the addition of 15 $\mu$M DCBQ, whereas the QA $-$closed condition was obtained by the addition of 5 $\mu$M DCMU in both pea thylakoid membranes and PSII samples. Closure of PSII center causes large changes in both demodulation and phase shift $\phi$. The results of multiplexponential model fits are shown in Table 1. In order to fit the data, a minimum of three exponential components is necessary at both $F_0$ (open centers) and $F_{max}$ (QA $-$closed centers) as judged by the low residuals (Fig. 2) and the low $\chi^2$ 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. 

Analysis with four decay components led to only a slight lowering of the $\chi^2$ and produced an additional component with an insignificant amplitude.

When QA 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 (QA $-$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 QA$^-$, 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 QA is reduced to QA$^-$ 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 and colleagues 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 QA$^-$/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.

of light, used here, also reduces all QA to QA− (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 QA 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 QA 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 (QA− closed), as expected.

PSII centers with double reduction of QA−

It appears that DCBQ opens most of the PSII centers by oxidizing the primary quinone acceptor QA. On the other hand, pretreatment with benzyl or methyl viologen and sodium dithionite and light leads to double reduction of QA− (see Van Mieghem et al.). Figure 4 shows the phase shift, Φ, and relative modulation, M, as a function of frequency in MHz in QA− and in doubly reduced QA− PSII centers in spin-
ach thylakoid membranes (Fig. 4A) and PSII membranes (Fig. 4B). Samples with doubly reduced QA have significantly different demodulation M and phase shift $\phi$ from those with singly reduced QA.

Parameters obtained by a triple exponential fit for the above fluorescence data are presented in Table 3.

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

|           | $\tau_1$ (ns) | $\tau_2$ (ns) | $\tau_3$ (ns) | $f_1$ | $f_2$ | $f_3$ | $\chi^2$ |
|-----------|---------------|---------------|---------------|-------|-------|-------|---------|
| QA-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 QA |
| 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 QA were prepared in the presence of sodium dithionite and methyl viologen plus laser illumination, and QA-closed PSII centers were obtained by simply illuminating samples with laser light. In this experiment, thylakoids were prepared from spinach.

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 QA, 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 QA-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) | $f_1$ | $f_2$ | $\chi^2$ |
|-----------|------------|------------|------------|------------|-------|-------|---------|
| QA-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 QA |
| PSII membranes | 0.77 | 0.76 | 1.00 | 1.00 | 1.31 | 3.48 |
| Thylakoid membranes | 0.57 | 0.70 | 1.00 | 1.00 | 8.51 |

*The samples with doubly reduced QA and those with reduced QA 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 QA, 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 QA), closed (all reduced QA) and those with doubly reduced QA. PSI1 centers in thylakoid membranes and PSII-enriched membranes, using multifrequency phase fluorometry. The fluorescence decay data were analyzed in terms of the usual multipexponential fluorescence decay and the alternate Lorentzian distribution functions. Multiplexponential analysis of QA-containing PSI1 centers showed three lifetime components: fast (40-140 ps), middle (400-600 ps) and slow (5-8 ns). Upon closure of the PSII centers (QA-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 that with reduced QA, the probability of charge separation in PSI1 decreases. The analysis with Lorentzian distribution functions, the focus of this paper, showed that in QA-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 QA, the lifetime peaks were shifted back to much lower lifetimes (0.57-0.77 ns) than those in samples with QA-closed centers.

Our results on thylakoids are in qualitative agreement with the reports by Haehnel et al. and by Keuper and Sauer. 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 PSI1 centers (QA-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 PSI1 (QA-closed) and PSI2 (QA-closed). The lifetime and the amplitude 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 PSI2 core and PSI1 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.) in the presence of DCBQ, which seems to keep most PSI1 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 PSI1 centers were closed. This provides strong evidence that the fast component is controlled by the process of energy conversion in the open PSI1 reaction centers. Schatz et al. showed that charge separation in PSI1 reaction centers is trap limited. This is consistent with a linear relationship between the total charge separation time and the antenna size (see Pearlstein). In open PSI1 centers the half-time of electron transfer from Pheo to QA is 300-500 ps. 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, Maurer et al. concluded that the long-lived (about 2 ns) fluorescence from closed PSI1 centers is recombination luminescence as proposed by Klimov et al. However, recent experimental results do not support the hypothesis of recombination luminescence in QA-containing PSI1. Such recombinational luminescence, however, occurs in PSI1 reaction centers devoid of QA.

In PSII samples that contain doubly reduced QA, lifetime distribution is shifted to shorter lifetimes from that in samples that contain reduced QA (Van Mieghem et al.; 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 QA 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 multitudes of conformational substates in a protein and by the dynamics of the protein (Gratton et al.). 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 QA-closed centers and centers with doubly reduced QA have provided a newer and alternate view on the changes that occur when PSI1 reaction centers are closed.

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