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## Chlorophyll a fluorescence lifetime distributions in open and closed photosystem II reaction center preparations

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We have measured the decay of chlorophyll a fluorescence at 4°C under anaerobic conditions in stabilized photosystem II reaction center complex isolated from spinach, using multifrequency (2–400 MHz) cross-correlation phase fluorometry. Examination of our data shows that although the fluorescence decay of open reaction centers (i.e., when both the electron donor P-680 and the electron acceptor pheophytin are capable of engaging in charge separation) can be analyzed as a multiexponential decay, another representation of the data is obtained when the decay is analyzed using a continuous distribution of lifetimes. Our results on the open reaction center differ from the two lifetime components of 25 ps and 35 ns published by Mimuro et al. (Biochim. Biophys. Acta 933 (1988) 478–486) for the D1-D2-cytochrome *b*-559 complex, obtained for F682 at 4°C by a time-resolved photon-counting spectrofluorometer. When the reaction centers are closed by pretreatment with sodium dithionite and methyl viologen followed by exposure to laser excitation, conditions known to result in accumulation of reduced pheophytin, a dramatic decrease in the contribution of the slow lifetime component(s) is observed. These results suggest that the slow distribution lifetime component(s) in the 5–20 ns range originate(s) in the back reaction of the charge separated state. On the other hand, the fast lifetime component(s) in the picosecond range may be only partially related to the charge separation, since no dramatic change is observed upon closure of the reaction center. Perhaps, this component is related, in part, to the excitation energy migration among the various chromophores in the reaction center preparations.

### Introduction

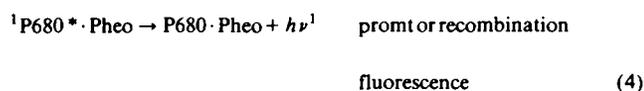
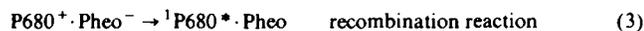
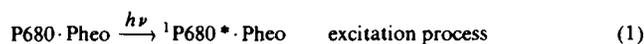
Primary photochemical reactions of photosynthesis, that begin with light absorption in the femtosecond time domain, involve a succession of temporal events: excitation migration among the various antenna pigment molecules, exciton trapping at the reaction center chlorophyll (or bacteriochlorophyll), primary charge sep-

aration, and stabilization of the stored energy [1–3]. A study of the molecular mechanism of charge separation and stabilization of the charge separated state, free from the extensive exciton migration process, becomes easier when isolated reaction centers are used. The isolation, crystallization, and the X-ray structure of the reaction center complex from the photosynthetic bacterium *Rhodospseudomonas viridis* [4–6] and *Rhodobacter sphaeroides* [7,8] have greatly aided in our understanding of the structure and function of this highly efficient, naturally occurring energy-converting device. Furthermore, these studies have also provided an incentive to study the plant reaction center from Photosystem II, the water-plastoquinone oxido-reductase, because of extensive similarities in their amino-acid sequences (see, for example, Ref. 9). Nanba and Satoh [10] have isolated a PS II reaction center complex from spinach containing three major proteins (D1, D2 and cytochrome *b*-559) and several chromophores (4 chlorophyll *a* and 2 pheophytin molecules). Recently, it also was shown to contain a small (4.8 kDa) polypeptide [11]. Unlike the bacterial reaction center, it does not contain bound

Abbreviations: *a*, amplitude of lifetime; *cG*[*n*], *cL*[*n*], center fluorescence lifetime of component *n* in ns in a Gaussian (G) or Lorentzian (L) shaped distribution of lifetimes; Chl, chlorophyll; *fG*[*n*], *fL*[*n*], fractional contribution of component *n* to the total intensity in a Gaussian or Lorentzian distribution of lifetimes; *F*, a representative expression of a fluorescence band, the maximum of which is located at wavelength  $\lambda$ , in nm; FWHM, full width at half maximum; P-680, primary electron donor of reaction center II; Pheo, pheophytin;  $\tau$ , fluorescence lifetime in ns; *wG*(*n*), *wL*(*n*), width of component *n* in ns in a Gaussian or Lorentzian distribution of lifetimes.

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quinones,  $Q_A$  and  $Q_B$  acceptors, or non-heme iron. Although quite unstable as isolated, it can be stabilized [12,13] and used to study the primary charge separation [14,15] and the primary back-reaction. The primary charge separation and recombination in the reaction center II can be written as (excluding reactions leading to heat losses):



where P680 is the primary electron donor and the ground state reaction center chlorophyll *a* (Chl *a*), Pheo is the primary electron acceptor pheophytin,  $h\nu$  is a photon or exciton,  ${}^1\text{P680}^*$  is excited singlet P680,  $\text{P680}^+$  is the oxidized reaction center P680 cation,  $\text{Pheo}^-$  is the reduced anion, and  $h\nu^1$  is an emitted photon (see also Ref. 13).

Wasielowski et al. [14,15] have measured directly the kinetics of primary charge separation at 4°C in stabilized complexes [12,13]: it occurs in  $3.0 \pm 0.5$  ps (Eq. 2). This is in good agreement with a 2.7 ps lifetime predicted by Schatz et al. [16] for charge separation from fluorescence data obtained from  $\text{O}_2$ -evolving *Synechococcus* particles. In the work of Wasielowski et al. [15], the formation of  ${}^1\text{P680}^+$  (Eqn. 1) was instrument-limited ( $< 500$  fs, the time of the laser flash), but its decay time matched the 3 ps rise time of  $\text{P680}^*$  (Eqn. 2). These kinetics are almost identical to the analogous reaction in photosynthetic bacterial reaction center [17–19]. Takahashi et al. [20] and Danielius et al. [21] have measured at 5°C a 32 ns lifetime for  $\text{P680}^+ \text{Pheo}^-$  (Eqn. 3) in the original Nanba-Satoh reaction-center preparations [10].

Chlorophyll *a* fluorescence is a non-destructive and a very sensitive intrinsic probe of the primary photochemical reactions of photosynthesis [22]. The decay of chlorophyll *a* fluorescence in photosynthetic systems has been described usually by a summation of several exponential components (see, for examples, Ref. 23). Using reaction-center preparations from Photosystem II [10], and a time-resolved photon-counting picosecond spectrofluorometer, Mimuro et al. [24] have shown that the decay kinetics of the fluorescence band at 682 nm ( $F_{682}$ ), suggested to originate in the reaction-center chlorophyll *a* P680, has two lifetime components at 4°C of about 25 ps and 35 ns. These times were suggested to correspond with the time of charge separation (Eq. 2) and charge recombination (Eq. 3 and 4). Using an

independent method, multifrequency cross-correlation phase fluorometry (see for example, Refs. 25–28), we show here that in open reaction centers, data can be represented appropriately by a continuous distribution of lifetimes in the picosecond to nanosecond range. Upon closure of the reaction center the slow components ranging from 5 to 20 ns decrease dramatically, suggesting that they originate from the recombination (back) reactions. A preliminary report of this observation was presented at a conference [29].

## Materials and Methods

### *Preparation and characteristics of stabilized D1-D2-cytochrome b-559 complex*

Stabilized Photosystem II (PS II) reaction center (RC) complex, containing D1, D2 and cytochrome (Cyt) *b*-559 proteins, was prepared from *Spinacea oleracea* (spinach) PS II at 4°C in dark from appressed membrane fragments [30,31] by a modification [12,13] of the original Nanba and Satoh method [10]. A 30 ml PS II membrane suspension, containing 1 mg/ml Chl, was solubilized in a mixture containing 4% (w/v) Triton X-100 and 50 mM Tris-HCl at pH 7.2 for 1 h with stirring. After this, the suspension was centrifuged at  $40000 \times g$  for 45 min, the supernatant was loaded onto a 15–1.6 cm column of TSK-GEL Toyopearl DEAE 650S (Supelco, Bellfonte, PA) pre-equilibrated with a mixture of 50 mM Tris-HCl (pH 7.2), 30 mM NaCl and 0.05% Triton X-100. The same pre-equilibration buffer was used to wash the column until the eluent  $A_{670}$  was 0.03; the RC fraction was eluted with a 30–200 mM NaCl gradient containing the same Tris/Triton buffer as above. The RC fraction was concentrated by treatment with slow addition of 32.5% (w/v) 3.35 kDa poly(ethylene glycol) (PEG) with stirring; after 1 h incubation without stirring, the suspension was centrifuged at  $40000 \times g$  for 15 min. The pellet then was resuspended in 50 mM Tris-HCl (pH 7.2) without added detergent; a quick (90 s) centrifugation of about 100  $\mu\text{g}$  Chl/ml reaction center suspension at  $1100 \times g$  removed colorless PEG aggregates from the sample. The RC was stored at  $-80^\circ\text{C}$  until use.

RC samples for fluorescence lifetime measurements were thawed in dim green light in a cold room and 0.04% Triton X-100 (final concentration) was added to keep the sample from aggregating. The samples were transferred rapidly to a nitrogen atmosphere and the following added quickly at final concentration in the order indicated: 20 mM glucose, 0.039 mg/ml catalase and 0.1 mg/ml glucose oxidase, as described earlier [13]. Photochemical activity of the RC preparation used in this study was 5000  $\mu\text{mol}$  silicomolybdate reduced per mg Chl per h, using diphenylcarbazide as electron donor. The absorption spectrum of the sample, measured before and after the fluorescence lifetime experi-

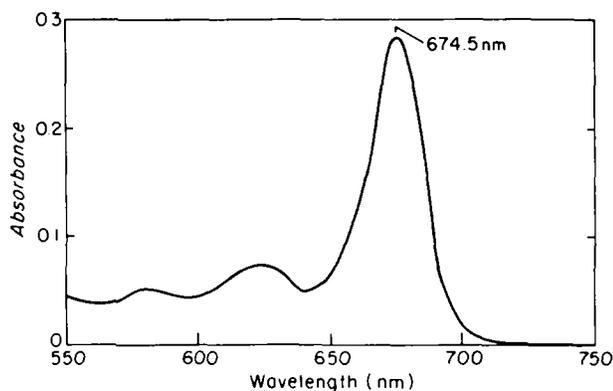


Fig. 1. Absorption spectrum, in the 550–750 nm range, of the reaction center (D1-D2-cytochrome *b* 559) complex at 4°C.

ment, showed a peak at 674.5 nm (Fig. 1), establishing its active nature [12,13]. The final absorbance of the sample was 0.3 at 674.5 nm in a 2 mm pathlength cuvette. To close the RC's (pre-reduce pheophytin so primary charge separation cannot occur) we included 1.5 mM freshly prepared sodium dithionite and 15  $\mu$ M methyl viologen in the resuspension buffer. Under these conditions, illumination (first with a standard flashlight and subsequently with 1.5 W 532 nm laser light for 2 min) reduces pheophytin to pheophytin<sup>-</sup>. Both the samples for the open and closed reaction centers were prepared and thereafter kept at 4°C until use.

#### Phase fluorometry lifetime measurements

One method to study the time-resolved fluorescence emission is to use multifrequency cross-correlation phase and modulation fluorometry [26–28]. This technique at present preferentially uses reliable, high-repetition-rate, pulsed light sources with high spectral intensity and purity, tunability, and high harmonic frequency content. For a comprehensive review see Gratton and Barbieri [32].

In the following we summarize the most important considerations. Assume that the light emission ( $I$ ) by a fluorescent sample upon delta-function excitation can be described as a superposition of exponential decays:

$$I(\lambda, t) = \sum_i a_i(\lambda) \exp(-t/\tau_i) = \sum_i (f_i(\lambda)/\tau_i) \exp(-t/\tau_i) \quad (5)$$

where the pre-exponential factor  $a_i(\lambda)$  is the contribution to the amplitude by the  $i$ -th component,  $\tau_i$  the fluorescent lifetime and  $f_i(\lambda) (= a_i \tau_i / \sum_j a_j \tau_j)$  the fractional contribution to the total steady-state intensity. In the frequency domain the fluorescence emission is characterized by the observables:  $\phi(\lambda, \omega)$ , the phase delay of the emission with respect to the excitation, and the modulation  $M(\lambda, \omega)$  upon excitation at a certain modulation frequency,  $\omega = 2\pi f$ , and wavelength,  $\lambda$ . The relative modulation,  $M(\lambda, \omega)$ , is defined as the ratio of the modulation of the fluorescence emission (em) to the

modulation of the excitation (ex),  $M_{em}/M_{exc} < 1$ . The phase  $\phi(\lambda, \omega)$  and modulation  $M(\lambda, \omega)$  are defined by:

$$\phi(\lambda, \omega) = \tan^{-1}[S(\lambda, \omega)/G(\lambda, \omega)] \quad (6)$$

$$M(\lambda, \omega) = [S^2(\lambda, \omega) + G^2(\lambda, \omega)]^{1/2} \quad (7)$$

where  $S(\lambda, \omega)$  and  $G(\lambda, \omega)$  are the normalized sine and cosine Fourier transforms of the fluorescence intensity  $I(\lambda, t)$ . For a multiexponential decay,  $S(\lambda, \omega)$  and  $G(\lambda, \omega)$  are:

$$S(\lambda, \omega) = \sum_i f_i [\omega \tau_i (1 + \omega^2 \tau_i^2)^{-1}] \quad (8)$$

$$G(\lambda, \omega) = \sum_i f_i (1 + \omega^2 \tau_i^2)^{-1} \quad (9)$$

The methodology and data analysis have been fully described earlier [33–35]. For a single exponential decay one can readily obtain the lifetime from Eqns. 6 and 7:  $\tau_\phi = \tau_M = \tan \phi/\omega$ . For most biological systems, however, a single exponential decay is not to be expected. Such systems are heterogeneous since a large number of microenvironments usually exist. Thus, one cannot expect that the fluorescence decay can be described by a well-defined set of a few lifetimes used in a discrete component analysis. To account for this, a new approach using discrete or continuous lifetime distribution functions using a sum of exponentials was recently developed by Alcalá et al. [36,37].

#### The instrument and the sample holder

The layout of the multifrequency cross-correlation phase fluorimeter used in our experiments is shown in Fig. 2. It is based on a modified version of previous instruments [27,28]. In the new version, 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 580 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 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. Radio frequency (RF) signals were obtained from a Marconi model 2022A signal generator and subsequently amplified by a Electronic Navigation Instruments model 603L RF power amplifier. The



TABLE I

A multiexponential fit for the fluorescence decay of open and closed reaction-center preparations

	$\tau_i$	$a_i$	$f_i$	Reduced $\chi^2$
Open	$0.32 \pm 0.02$	0.89	$0.33 \pm 0.01$	6.2
	$3.15 \pm 0.27$	0.10	$0.37 \pm 0.02$	
	$27.00 \pm 2.47$	0.10	0.30	
Closed	$0.35 \pm 0.01$	0.88	$0.50 \pm 0.01$	1.14
	$2.20 \pm 0.13$	0.11	$0.41 \pm 0.01$	
	$16.00 \pm 2.54$	0.003	0.09	

and the reduced  $\chi^2$  values (Table I). A double- or single-exponential fit is not sufficient to describe the data. The fluorescence lifetimes,  $\tau_i$ , are given in ns, the fraction intensities are denoted by  $f_i$ , while the amplitudes are given by  $a_i$ . The three lifetime components in the open reaction centers are approx. 320 ps (33%, fractional intensity), 3.2 ns (37%) and 27 ns (30%). Upon closure of the reaction centers, the fluorescence lifetimes and fractional intensities become: 350 ps (50%), 2.2 ns (41%) and 16 ns (only 9%). Thus, the most dramatic change is clearly a decrease in lifetime of the slow component with a concomitant decrease in its fractional intensity. These values differ somewhat from the values obtained by Mimuro et al. [24] at 4°C for apparently similar RC complexes which, however, contained some unbond Chl. They found no dependence of the fluorescence decay parameters on the emission wavelength in the range from 670 to 690 nm. They obtained two lifetime components with values of about 25 ps and 35 ns, tentatively assigned to the kinetics of the charge separation and the charge recombination, respectively.

#### Analysis of the data: Lifetime distributions

In view of the fact that a more realistic representation of the data has been shown to exist for even far less complicated systems, we analyzed the same data with Lorentzian and Gaussian lifetime distribution functions [36,37]. Since these fitting procedures may converge very slowly, the end results of a uniform distribution function analysis were used as starting values for the Gaussian and Lorentzian cases. The solid lines in Fig. 4 present the results for a continuous distribution of lifetimes. The residuals obtained from these fits show no specific frequency dependence; they are randomly distributed, indicating an adequate quality of the fit to the data.

The actual lifetime distributions (for the Lorentzian case) are shown in Fig. 5A and 5B for the open and closed reaction center preparations, respectively. Deviations, on an enlarged scale, are shown in Fig. 6A and 6B for the open and closed reaction center preparations, respectively. Comparison of the results obtained from the analysis using these lifetime distribution functions show that in open reaction centers (Fig. 5A) a high fraction of fast components are clustered in the 0–300 ps range. This is followed by a decay to slow components in the 2–20 ns region. Upon closure of the reaction center (Fig. 5B), a dramatic change occurs: a shift from the very broad, almost featureless, distribution to a narrower one with an increased fractional contribution from the shorter lifetimes. The most dramatic effect is the disappearance of the long-lived slow (5 to 20 ns) fluorescence components upon closure of the reaction centers. Since we interpret the closure phenomenon as the reduction of Pheo to Pheo<sup>-</sup> [15], the disappearance of these slow components is interpreted to be

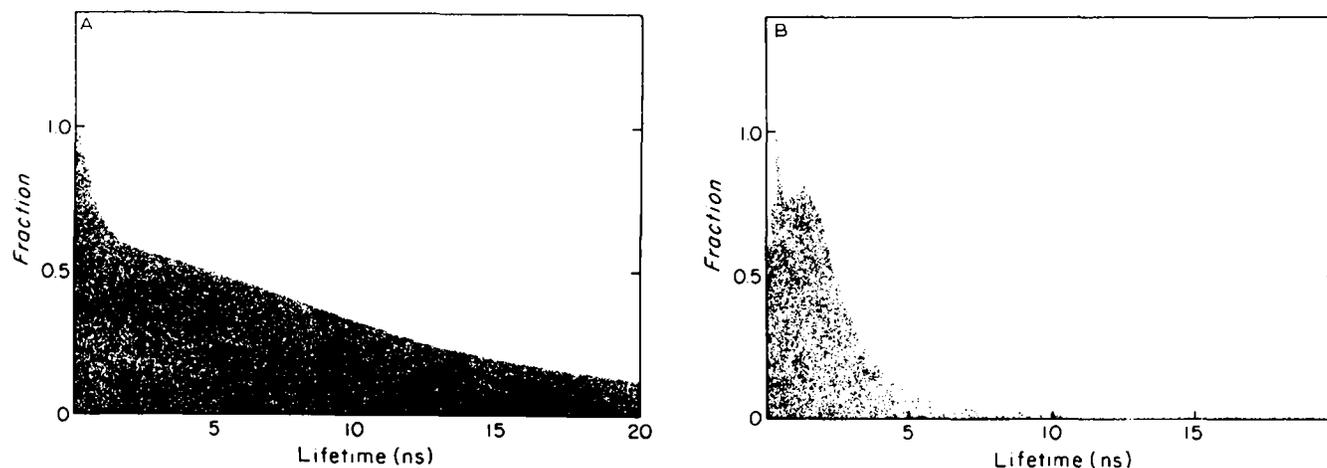


Fig. 5. Lifetime distribution (fraction versus lifetime) for the open (A) and closed (B) reaction center (D1-D2-cytochrome *b* 559) complex (Lorentzian case) at 4°C. Calculated values for A (open reaction centers) are:  $cL[1] = 0.000$ ;  $wL[1] = 1.291$ ;  $fL[1] = 0.469$ ;  $cL[2] = 1.768$ ;  $wL[2] = 19.847$ ;  $fL[2] = 0.531$ ; and reduced  $\chi^2 = 19.995$ . Calculated values for B (closed reaction centers) are:  $cL[1] = 0.340$ ;  $wL[1] = 0.050$ ;  $fL[1] = 0.355$ ;  $cL[2] = 1.299$ ;  $wL[2] = 2.923$ ;  $fL[2] = 0.634$ ; reduced  $\chi^2 = 4.423$ . See list of abbreviations for the symbols.

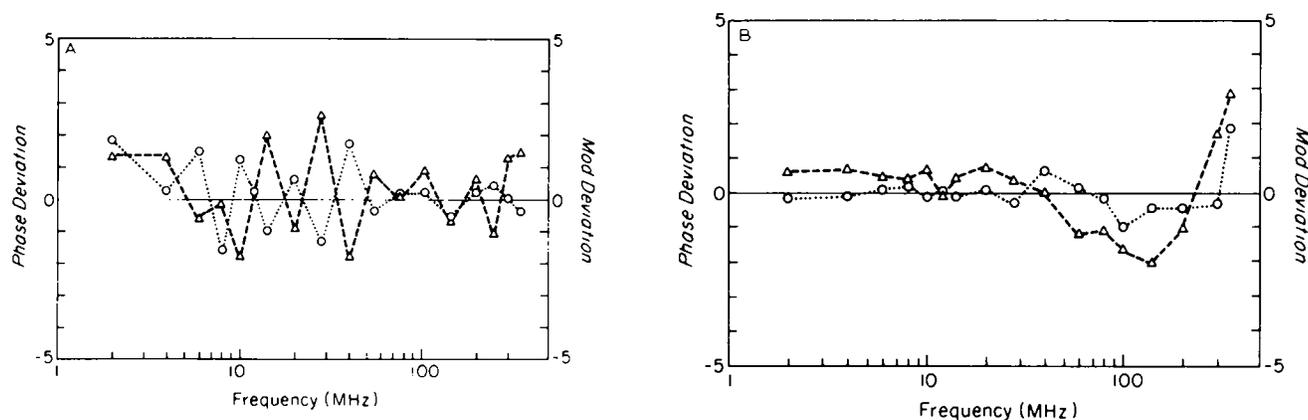
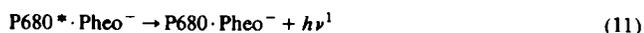


Fig. 6. Deviations in the data with the calculated and the experimental results in Fig. 5, plotted on an expanded scale, are shown for the open reaction centers (A) and closed reaction centers (B).

due to the absence of the recombination reactions (cf. with Eqns. 1–4):



Thus, the slow components (5–20 ns) here are associated with the recombination reactions, as suggested by Klimov and co-workers (see Klimov and Krasnovsky, Ref. 38) for variable Chl *a* fluorescence in PS II. On the other hand, the fast components (0–300 ps) are associated with the prompt fluorescence that may or may not include contributions for excitation energy transfers among the 6 chromophores (4 Chl *a* and 2 Pheo molecules) and competition with charge separation. In view of the fact that no dramatic change occurs in the lifetime of the fast components upon closure of the reaction center, although one can see a more pronounced sharpening apparently caused by a larger contribution of the shorter component to the total distribution, it is considered likely that charge separation does not influence these components very much.

### Concluding remarks

The above results and conclusions differ somewhat from the 25 ps and 35 ns components, tentatively assigned to charge separation and charge recombination reactions, respectively (Mimuro et al., Ref. 24).

One has to keep in mind that the analysis of the fluorescence decay in terms of a discrete set of two or three components is an approximation due to the resolution of the instruments used and assumes unique environs of the emitting species which are stable over the fluorescent lifetime. With the ever increasing instrument accuracy, the number of retrievable components has steadily increased over the years. The matching of a certain lifetime with a certain conformational state usu-

ally lacks the justification from an appropriate physical model and seems to be an oversimplification.

It was shown for a number of proteins that a continuous distribution of lifetimes better approximates the fluorescence decay. Two explanations were proposed [39]. A static origin in which a unique fluorescence lifetime can be associated with every element of a distribution of conformational substates. The rate of interconversion between substates has to be much slower than the decay rates. The other possibility is a dynamic origin. Collisions, excitation energy transfer, dipolar relaxation can alter the decay of an excited state. The dynamics of a system determines the interconversion between conformational substates. A justification for the use of lifetime distribution functions in the analysis of the fluorescence decay can be inferred from the studies performed on single tryptophan proteins [see, for example, Refs. 36,37,39]. The existence of a continuous lifetime distribution was observed, giving support to the idea of many, almost degenerate conformational substates. Data could not be fitted to a bimodal distribution which would indicate the existence of two well-defined local environs the fluorescent tryptophan caused by its different conformations. This finding undermines the idea of linking a certain lifetime value to a specific conformational (sub)state. These arguments lend support to the idea that the lifetime distribution functions, as presented in this paper, present a more realistic representation of the data.

It is worth noting that the three exponential component analysis and the distribution analysis provide qualitatively similar results. In fact, both analyses show that the region of short lifetime value is little changed between the closed and open centers. Instead the long lifetime region shows dramatic changes in both analyses. The discrete component analysis place that in the intermediate lifetime region: there is a shifting of the lifetime value to shorter decay times upon closure of the RC. In analogy, the distribution analysis shows a peak-

ing of the distribution in the 1–3 ns region. We believe that in the presence of a lifetime distribution, the exponential analysis represents a sampling of the distribution providing values which best reproduce the density of lifetime components. In that respect both analyses are in good agreement.

Our conclusion about charge recombination of the radical pair ( $P680^+Pheo^-$ ) in isolated PS II reaction center is not in agreement with that of Barber et al. [40]. However, during the revision of our paper, we became aware of a recent paper by Crystall et al. [41] which now suggests that charge recombination represents a large function of the measured emission in D1-D2-Cytochrome *b* 559 reaction center complex stabilized by the techniques of McTavish et al. [13]. Our conclusions are qualitatively (though not quantitatively) in agreement with Ref. 41. However, no attempt was made by Crystall et al. to show the disappearance of recombinational emission upon prereduction of pheophytin, an experiment that proves the validity of our conclusion (cf. Fig. 5A and 5B of this paper).

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