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# Global and target analysis of fluorescence measurements on photosystem 2 reaction centers upon red excitation

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We measured the excited state dynamics of the photosystem 2 reaction center (RC) by fluorescence measurements with good temporal and spectral resolution and a high signal to noise. The fluorescence decay is highly multi-exponential. Upon excitation at 681 nm the data are well described with lifetimes of 6, 34, 160 ps and 7 ns. The 6 ps Decay Associated Spectrum (DAS) is red shifted with respect to the long lived DAS whereas the 34 ps DAS is blue shifted. The 34 ps component is dominant over the 6 ps component. More importantly, we show that the observed intermediate lifetimes (30–50 ps) cannot be ascribed to radical-pair relaxation only, since they correspond with significant spectral evolution of the fluorescence. From a target analysis with a minimal model, involving two radiative and three dark compartments, we conclude that these data are consistent with a kinetic scheme including fast charge separation (1 ps), moderately slow energy transfer within the RC, and radical pair relaxation.

# 1. Introduction

The recent progress<sup>1-3</sup> in resolving the structure of the plant Photosystem 2 (PS2) core complex (consisting of the reaction center and the two core antenna complexes CP43 and CP47) revitalizes the study and understanding of the energy trapping and charge separation dynamics in the isolated RC complex, which consists of 6 Chl a and 2 Pheo a pigments. On top of the structural data a wealth of biochemical work now allows for the assignment of the excited state energies of the different pigments of the reaction center.<sup>4,5</sup> The initial kinetics in the reaction center of PS2 has been studied in several groups, the excited-state decay and radical-pair formation kinetics are highly multi-exponential, most studies report lifetimes which range from sub-picosecond to tens of picoseconds.<sup>6-11</sup> The differences in the reported results have decreased but the agreement about the interpretation of the data is not complete. A central issue here is: what is the intrinsic rate of charge separation, reported values range from sub ps<sup>8</sup> to values between 3<sup>7</sup> and 20 ps.<sup>6</sup> Especially the discrimination between energy transfer and electron transfer processes is not trivial. This is mostly caused by the congested nature of the  $Q_v$  region of the spectrum. Moreover, despite the absence of a secondary electron acceptor, the observed dynamics stretch out into the nanosecond timescale, *i.e.* they are not limited to the time scale associated with the initial charge separation process, and are due to relaxation processes occuring within the radical pair states. Already the first occurrence of a charge-separated state, as measured in the Pheophytin  $Q_x$  region, is very multi-exponential.<sup>6</sup> Two mechanisms have been invoked to explain this. The multi-exponential kinetics could be due to slow energy-transfer processes to P680, or the kinetics could be due to fast relaxation between radical-pair states, i.e. the question amounts to whether radical pair relaxation processes occur on the timescale of the initial electron transfer process ( $\approx 10$  ps) or if the observations are due to slow energy transfer.

The multiple radical-pair model predicts little or no changes in the emission spectrum after ultra-fast equilibration. In the case of energy-transfer limited charge-separation spectral changes can occur because slow transfer limits complete equilibration. Here we attempt to discriminate between these two models by looking at the changes in the fluorescence properties on a picosecond timescale. For this we employ a synchroscan streak-camera system that combines good time-resolution with good spectral resolution. With this set up, a dominant lifetime of 1 ps can reliably be estimated.<sup>12,13</sup> Moreover, because of our combined spectral and temporal resolution we are able to apply computational methods that resolve the fluorescence signal and the scattered excitation light. Therefore we could measure the fluorescence dynamics while exciting in the (extreme) red part of the absorption spectrum.

# 2. Materials and methods

## Sample preparation

PS2 RCs were purified from spinach Tris-washed "BBY" grana membranes as described by van Leeuwen *et al.*<sup>14</sup> In these preparations the secondary quinone acceptor is lost, so the electron transfer process does not go beyond the formation of the state P680<sup>+</sup>Pheo<sup>-</sup>, from which the P680 triplet state is formed in about 30 ns.<sup>15</sup> Oxygen free conditions were maintained during the experiments by the addition of the enzymatic oxygen-consuming system of glucose-oxidase, catalase and glucose in MES buffer.

## **Fluorescence experiment**

Fluorescence experiments were performed on PS2 reaction centers employing a multiwavelength synchroscan streak camera detection system synchronized to an amplified (repetition rate 50 kHz) Ti:Sapphire laser system. The instrument records emission spectra over a large spectral window (300 nm) in

parallel, with a temporal instrument response of  $\approx 3$  ps full width at half maximum (FWHM). The fluorescence was dispersed before the streak camera using an f = 1/4m imaging spectrograph with a 50 lines mm<sup>-1</sup> grating. Spectral resolution was 5 nm. The spectrograph caused only limited broadening of the response function from 1.8 ps to  $\approx$  2.8 ps. Slow drift of the synchronization in combination with data collection times of  $\approx 20 \text{ min frame}^{-1}$  gave rise to an instrument response function (IRF) of 3–3.5 ps FWHM, as deduced from the width of the scattering peak for scan lengths of 200 ps. A scan length of 800 ps or 2 ns increased the IRF width to  $\approx 8$  or  $\approx 20$  ps FWHM. The images were corrected for wavelength-dependent temporal shifts caused by dispersion in the light-collecting optics, the spectrograph and distortions in the streak-tube, by recording the IRF using scattered white-light pulses with well-defined chirp, as measured using the optical Kerr signal in  $CS_2$  to simulate the emission from the sample. The experiments were performed in a spinning cell rotating at a speed of 600 rpm. Typical excitation densities of < 10 nJ pulse<sup>-1</sup>, in combination with a spot size of  $\approx 0.2$  mm ensured annihilation free conditions.<sup>16</sup> Absorption spectra were recorded of the samples before and after the experiments, and showed no indication for sample degradation. Typical data sets as presented here are the average of up to 20 streak images obtained after 20 min of data collection per image. For excitation wavelengths overlapping with the emission spectrum the polarization of the excitation light was horizontal whereas the vertically polarized fluorescence was detected. Using this perpendicular geometry the amount of scattered excitation light was strongly reduced, to a degree that it did not affect the analysis of the data.

We are making a systematic error in our experiments because we are not measuring at the magic angle, however the effect of this procedure on the apparent lifetime of the initially excited state should be very limited, especially if one takes into account the fast depolarization reported in ref. 17

Due to the fact that the streak camera scans back and forth at a repetition rate of 76 MHz long-lived fluorescence is recorded during the back sweep (and during the next sweep), so the signal (see Fig. 1) shows a significant amount of fluorescence before the excitation pulse; this is taken into account by the analysis program<sup>12</sup> and enables estimation of one (average) long lifetime.

#### Global and target analysis

In global analysis a model consisting of independent exponential decays is fitted to the data  $\psi(\lambda, t)$ 

$$\psi(\lambda, t) = \sum_{i=1}^{n_{\text{comp}}} c_i(t) \text{DAS}_i(\lambda)$$

where  $n_{\rm comp}$  is the number of components,  $c_i(t)$  corresponds to the convolution of the *i*-th exponential decay with the IRF,<sup>12</sup> and DAS<sub>i</sub>( $\lambda$ ) is the *i*-th Decay Associated Spectrum. In target analysis<sup>18</sup> a compartmental scheme is used to describe the concentrations of the compartments (states). Transitions to and from compartments are described by microscopic rate constants. The model that is fitted to the data now reads

$$\psi(\lambda, t) = \sum_{i=1}^{n_{\text{comp}}} c_i(t) \mathbf{SAS}_i(\lambda)$$

where  $c_i(t)$  now corresponds to the concentration of the *i*-th compartment. SAS<sub>i</sub>( $\lambda$ ) is the *i*-th Species Associated Spectrum. Note that components that do not emit light will not contribute to this sum. However, their presence can affect the complicated multiexponential decay of emitting components. The concentrations of all compartments are collated in a vector  $c(t) = \lfloor c_1(t) \ c_2(t) \ \dots \ c_{n_{comp}}(t) \rfloor^T$  which obeys the differential



Fig. 1 Selected traces of PS2RC excited at 681 nm. Data consisted of 24 traces between 640 and 760 nm. Data from time bases of 200 and 800 ps have been scaled and superimposed. Global analysis using four exponential decays resulted in lifetimes of 6, 34 ps, 0.16 and 7 ns. Fit is shown as dotted (200 ps time base) or dashed (800 ps time base) lines, residuals are depicted in the insets. The time base from -10 to +10 ps (relative to the maximum of the instrument response) is linear, and from 10 to 1000 ps and before -10 ps is logarithmic.

equation

$$\frac{\mathrm{d}}{\mathrm{d}t}c(t) = Kc(t) + j(t)$$

where the transfer matrix K contains off-diagonal elements  $k_{pq}$ , representing the microscopic rate constant from compartment q to compartment p. The diagonal elements contain the total decay rates of each compartment. The input to the compartments is  $j(t) = \text{IRF}(t) \lfloor x_1 x_2 \dots x_{n_{\text{comp}}} \rfloor^T$ .

### 3. Results

Fig. 1 shows representative traces extracted from the Streak Images of PS2 measurements on a 200 ps and an 800 ps time base, excitation was at 681 nm (fwhm  $\approx 6$  nm). Since both datasets, each consisting of 24 traces between 640 and 760 nm, show an identical trend a simultaneous global analysis was performed taking into account the different IRF widths and different amounts of scattered excitation light with the different time bases, which are both clearly visible at 681 nm. A global analysis describes the spectral and temporal evolution in the data without restriction to a specific physical model, resulting in estimated lifetimes and decay-associated spectra (DAS). The multiexponential fluorescence decays are adequately fitted

(dotted and dashed lines in Fig. 1) with four exponential decays and a scatter component. Obviously, isolating the scatter contribution is crucial for obtaining a reliable fit of the initial kinetics. We found that by restricting the wavelength range for the pulse limited component (scattered excitation light) to the region of the excitation pulse we could get reliable results. By comparing different datasets with variable amounts of scattered light, we found that the other lifetimes from the fit were not affected by the intensity of the scatter artefact. The chaindashed curve in Fig. 2A represents this pulse scattering contribution. The first DAS (6 ps, solid line in Fig. 2A) is clearly red shifted compared to the long lived DAS (7 ns, dot dashed) which is of similar shape as the 0.16 ns DAS (dashed). Remarkably, the largest DAS (34 ps, dotted) is blue shifted. Taking into account a data set collected up to 2.2 ns did not alter these results.

A global analysis of a data set collected using 690 nm excitation (200 ps time base) yielded components of 9, 51 ps and 7 ns, their DAS spectra are shown in Fig. 2B as solid, dotted and dot-dashed curves, respectively. Here also the first DAS is redshifted, whereas the second DAS is blue shifted relative to the long lived DAS.

# 4. Discussion

The goal of this work was to be able to resolve excited-state equilibration processes and electron transfer processes, by performing a target analysis incorporating both features. Ideally this corresponds to a model containing at least eight chlorin excited states plus three dark radical pair states. This would correspond to eleven lifetimes, which obviously is not feasible. Therefore we commence with a brief discussion of the estimated DAS and lifetimes. The fast component of 6-9 ps, with a DAS that is red-shifted relative to the DAS of the slower components, has the signature of charge separation from the directly excited active branch. At this point however, the exact nature of this process is still open: it could be direct charge separation from P680; the decay of an equilibrium between P680\* and a sub-ps-formed charge separated state into a more effectively trapped, relaxed, radical pair state; or decay of a (sub-ps) equilibrated state between P680\* and Chl\*, or there could even be multiple pathways for charge separation.<sup>19</sup> The intermediate lifetime of 30-50 ps has a DAS that is slightly blue-shifted relative to the long-lived DAS, therefore it may not represent the decay of an equilibrated RC. This



**Fig. 2** DAS from global analysis of PS2RC excited at 681 (A) or 690 (B) nm. Key: (lifetimes and line types) (A) 6 (solid), 34 (dotted) ps, 0.16 (dashed) and 7 ns (dot-dashed), scatter (chain-dashed). (B) 9 (solid), 51 (dotted) ps, and 7 ns (dot-dashed), scatter (chain-dashed). Vertical bars indicate estimated standard error.

observation suggests that the corresponding components (with lifetimes of 20–50 ps) observed in pump–probe experiments are not solely due to radical-pair relaxation. The excitation conditions are such that there cannot be any significant direct excitation of the blue pool of pigments. A significant population of blue pigments results from energy transfer from a red state, and from P680 in equilibrium with a radical pair state, to several blue-absorbing states.

These results can be explained by two different models which have in common that all red states of the PS2RC occur within the central 6 chlorins of the RC,<sup>20</sup> that the energy differences between P680\* and the first radical pair state are not very large, and that there are multiple radical pairs. In the first model, all energy transfer steps within the RC are thought to occur within a few ps.<sup>17</sup> The blue shift of the 30-50 ps phase compared to the 6 ps phase must in this case be explained by energy transfer from the central chlorins to the peripheral chlorophylls, which in this representation absorb at around 670 nm. In the second model, slow energy transfer between two red states via blue pigments is assumed, together with trapping in a charge separated state from one of these red states.<sup>8</sup> The energetic aspects of this model may be in line with work that showed that PA (in Synechocystis 6803) is blue shifted compared to the other central pigments of the RC.<sup>21</sup> A population of these blue states may then contribute to the observed blue shift. A point of concern of the first model is that the observed blue shift of the 30-50 ps phase may be larger than expected from the population of the peripheral chlorophylls, also because the first radical pair relaxation process probably occurs with about the same kinetics. A point of concern of the second model is that within the newest structures<sup>3</sup> no good evidence for slow energy transfer within the central RC pigments can be found. If one accepts the notion that energy transfer from the peripheral chlorophylls to the central RC pigments, with minimal centerto-center distances of about 23–24 Å and an unfavourable orientation factor for energy transfer,<sup>22</sup> occurs in 20–30 ps, then it is hard to realize that within the central RC pigments, with center-to-center distances of 10-11 Å and more favourable orientation factors, also energy transfer processes of 20-30 ps will occur. A second point of concern is that there are indications that PA absorbs more to the red in plant PS2 than in cyanobacterial PS2, because the electrochromic shifts that led to the attribution of the absorption maximum of  $P_A$  at 672.5 nm in Synechocystis 6803<sup>21</sup> give a maximum of 678 nm in PS2 membrane fragments from spinach.<sup>23</sup>

Below we will discuss the results from a target analysis<sup>7,18,24</sup> on the data. Such an analysis describes the data as originating from a compartmental model and directly estimates the spectra of- and transfer rates between- the compartments. These compartments can then be interpreted as representing individual pigments or groups of pigments, depending on the level of sophistication of the model. A minimal number of two excited state compartments were chosen. We also tried with three, but this appeared unfeasible. These two excited state compartments can represent chlorins directly involved in charge separation (compartment 2) and chlorins not directly involved in charge separation (compartment 1). These can be H<sub>A</sub> and B<sub>A</sub> (compartment 2) and H<sub>B</sub> and B<sub>B</sub> and also all blue-absorbing chlorophylls (compartment 1), or alternatively, all six central chlorins (compartment 2) and the two peripheral chlorophylls (compartment 1). Since there are two long-lived components of 0.16 and 7 ns, and these can be safely assigned to radical pair kinetics, we need to include at least two radical pair states in the model. We chose to add a third sequential radical pair state. The compartmental model is depicted in Fig. 3. The first two compartments are excited states, and the relative population is taken to be 50%/50% with 681 nm excitation, whereas with 690 nm excitation it is a free parameter. For the RP states, either the forward and backward rates can be estimated independently, or the forward rate can be estimated together



Fig. 3 Compartmental model for the RC. Charge separation starts from the chlorines in compartment 2. Compartment 1 represents the other chlorines in the RC. Compartments 3, 4 and 5 represent radical pair relaxation

with a free energy difference. We chose the latter. The fluorescence rate was fixed at  $(5 \text{ ns})^{-1}$ . Thus the simple scheme depicted in Fig. 3 has five free rate parameters, four free equilibrium parameters, and an excited state population parameter. This adds up to ten kinetic parameters, which is much more than the four lifetimes estimated in the global analysis. Now by using only two Species Associated Spectra (SAS), because the SAS of the three radical-pair states are zero, three more kinetic parameters can be estimated. We chose starting values for the kinetic parameters with very fast charge separation, in accordance with the pump-probe measurements discussed in the introduction. Table 1 collates the parameters of the compartmental model. All kinetic parameters could be estimated, albeit with large estimated standard errors. The estimated SAS with 681 nm excitation are shown in Fig. 4A. The second SAS (dotted), which belongs to the compartment from which charge separation starts, is slightly red shifted with respect to the first SAS. With the 690 nm excited data set again the second SAS (dotted in Fig. 4B) is red-shifted. The amplitudes of each compartment's population are collated in Tables 2 and 3. Note that these emission data are consistent with the presence of a lifetime of 1 ps, with an amplitude



Fig. 4 SAS from target analysis of PS2RC excited at 681 (A) or 690 (B) nm. Key: compartment 1 (solid), compartment 2 (dotted), scatter (dashed). Vertical bars indicate estimated standard error.

comparable to that of other fast lifetimes (6-10 ps). The first compartment, consisting of chlorins not directly involved in charge separation, decays mainly with a lifetime of 36 or 45 ps, thus explaining the blue shifted DAS of Fig. 2. The energy transfer between compartments 1 and 2 is a free parameter, which in both cases was estimated to be about 26 ns<sup>-1</sup>, thus these emission data are consistent with the presence of slow ET within the RC.8

With the present method the spectral resolution enables the resolving of the scattered excitation light (characterized by the IRF time profile and the spectrum of the excitation pulse) from the fast chlorin emission. (Integration across wavelength would compromise this.) With our IRF width of  $\approx 3$  ps, and the best achievable signal to noise ratio the global analysis (Fig. 2) does not provide evidence for a dominant sub-ps emission component. This observation contradicts the prediction of a dominant <3 ps component in,<sup>7</sup> which was based upon SPT measurements with an IRF width of  $\approx 30$  ps. The target analysis which uses two SAS and five lifetimes enables resolution of the amplitude of a (sub)ps component with a chlorin spectrum. According to Tables 2 and 3 the amplitude of this 1 or 0.7 ps component is equal to that of the 6 or 10 ps component. Thus these data indicate that a (sub)ps component would not be dominant. But this is a model dependent

**Table 1** Parameters of the compartmental model for the RC shown in Fig.  $3^a$ 

λexc	$\Delta_{12}/meV$	$\Delta_{23}/meV$	$\Delta_{34}/meV$	$\Delta_{45}/meV$	$k_{21}/{\rm ns}^{-1}$	$k_{12}/{\rm ns}^{-1}$	$k_{32}/{\rm ns}^{-1}$	$k_{23}/{\rm ns}^{-1}$	$k_{43}/{\rm ns}^{-1}$	$k_{34}/{\rm ns}^{-1}$	$k_{54}/{\rm ns}^{-1}$	$k_{45}/{\rm ns}^{-1}$	$k_5/\mathrm{ns}^{-1}$	$k_{\rm fl}/{\rm ns}^{-1}$
681 nm 690 nm	$\begin{array}{c} 7\pm7\\ 4\pm4 \end{array}$	$\begin{array}{c} 0 \pm 20 \\ 0 \pm 20 \end{array}$	$\begin{array}{c} 35\pm5\\ 65\pm30 \end{array}$	$\begin{array}{c} 17 \pm 20 \\ 31 \pm 20 \end{array}$	$\begin{array}{c} 26 \pm 1 \\ 24 \pm 3 \end{array}$	19 21	$\begin{array}{c} 500 \pm 120 \\ 670 \pm 300 \end{array}$	510 670	$\begin{array}{c} 240\pm30\\ 160\pm40 \end{array}$	61 13	$\begin{array}{c}5\pm3\\2\pm2\end{array}$	2.4 0.7	$\begin{array}{c} 0.2\pm0.2\\ 0.2\pm0.2 \end{array}$	0.2 0.2
<sup><i>a</i></sup> Estimated errors are given after $\pm$ symbol. Other parameters are fixed or derived.														

Table 2	Amplitude	matrix fo	or the	different	lifetimes,	681	nm excitation <sup><i>a</i></sup>	
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Compartment	1	2	3	4	5
Excitation	0.50	0.50	0	0	0
1 ps	0	0.19	-0.23	0.05	0
6 ps	-0.02	0.19	0.13	-0.30	0.01
36 ps	0.39	-0.04	-0.06	-0.36	0.06
0.2 ns	0.08	0.09	0.09	0.33	-0.60
8 ns	0.05	0.07	0.07	0.28	0.53

 Table 3 Amplitude matrix for the different lifetimes, 690 nm excitation<sup>a</sup>

Compartment	1	2	3	4	5
Excitation	0.13	0.87	0	0	0
0.7 ps	-0.01	0.39	-0.43	0.05	0
10 ps	-0.11	0.39	0.35	-0.65	0.01
44 ps	0.18	0.02	0.01	-0.22	0.02
0.4 ns	0.05	0.05	0.05	0.60	-0.77
17 ns	0.02	0.02	0.02	0.22	0.74
<sup><i>a</i></sup> Note that the sum of a	all amplitudes equals the e	citation of each compar	tment.		

observation. Direct evidence could in principle be obtained from a spectrally resolved upconversion experiment. Although such an instrument possesses an IRF width of  $\approx 0.2$  ps,<sup>25</sup> it is not clear whether annihilation free measurements are feasible.

The estimated parameters in Table 1 appear reasonable. However, in view of the large estimated standard errors, and of the simplified compartmental scheme, we refrain from a more elaborate discussion of the estimated values. Instead we aim to combine the information from these emission data with dispersed sub-ps time resolution infrared difference absorption measurements, which provide more clues for resolving the different chlorins.<sup>26</sup>

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