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Picosecond fluorescence kinetics of the D_1-D_2-cyt-*b*-559 Photosystem II reaction center complex. Energy transfer and primary charge separation processes

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The fluorescence kinetics of the D_1-D_2-cyt-*b*-559 Photosystem II reaction-center complex have been characterized in the picosecond and nanosecond time ranges. Measurements have been performed in the presence of β-lauryl maltoside under anaerobic conditions, both at 277 K and 77 K. Global analysis of decays recorded at different wavelengths and with different time resolutions yielded the decay-associated emission spectra of six exponential lifetime components, in the range of 1–6 ps up to 35 ns. We report here an ultrafast ($\tau \approx 1$–6 ps) fluorescence lifetime component with dominant amplitude ($\geq 90\%$), which is thought to reflect the primary charge separation process. Furthermore, we have resolved a component with a 30–40 ps lifetime, its DAS indicating energy transfer. The lifetime of the primary radical pair $P_{680}^{+}I^{-}$, reflected by its recombination fluorescence, was found to be temperature-dependent. At 277 K this lifetime was 30 ns with a relative yield of 0.87, whereas at 77 K the radical-pair lifetime is increased to 35 ns with a relative yield of only 0.24 (both yields are corrected for the emission from uncoupled chlorophylls).

Introduction

Since the reaction-center complex of Photosystem II (PS II) from higher plants has been identified as the D_1-D_2-cyt-*b*-559 pigment protein complex [1,2], a variety of spectroscopic studies have been performed in order to characterize its pigment composition and organization as well as its primary photochemistry. The pigment content of this complex was initially characterized as 4–5 chlorophyll *a* (Chl *a*) molecules, 2 pheophytin *a* (I), 1 β-carotene (Car) and 1–2 cytochrome *b*-559 (cyt) molecules [1,2]. This pigment composition would be analogous to that found in reaction centers of purple bacteria [3,4]. The quinone electron acceptor Q_A has been lost, however. More recently, the actual pigment composition of the D_1-D_2-cyt-*b*-559 reaction-center preparations has become a subject of discussion, since the number of Chl *a* molecules per reaction center has been found to vary with the different preparation methods between 4 [5] or 6 [6] and 10–12 [7]. Furthermore, it has been argued that the use of Triton X-100 as a solubilizing agent should lead to partial denaturation of the pigment protein complexes, as compared to the use of β-lauryl maltoside [7]. While maintaining the same protein composition, the use of Triton X-100 caused the loss of some three Chls [7]. Previously, it has been shown that the rather limited stability of the D_1-D_2-cyt-*b*-559 complex isolated with Triton X-100 can be drastically improved by the subsequent substitution of Triton X-100 by β-lauryl maltoside [8]. Other factors that improve

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Abbreviations: PS II, Photosystem II; P_{680}, primary donor of Photosystem II; I, pheophytin *a*; Chl, chlorophyll *a*; cyt, cytochrome; FWHM, full width at half maximum; DAS, decay-associated spectrum.

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the stability of these reaction-center complexes are precipitation with poly(ethylene glycol) [9], and anaerobic conditions [9,10].

The primary photochemistry occurring in the D_1-D_2-cyt-*b*-559 complex is thought to consist of a light-induced charge-separation process between the excited primary donor P^*_{680} (presumably a Chl *a* dimer [11]) and the primary acceptor, I (pheophytin *a*). As the secondary acceptor (quinone Q_A) is lost, the corresponding charge stabilization step cannot take place and the primary radical pair $P^+_{680}I^-$ recombines. This charge recombination can, inter alia, lead to a repopulation of the excited state of the donor, P^*_{680}, thus giving rise to the so-called recombination fluorescence. Transient absorption measurements in the nano- and picosecond time ranges revealed a lifetime of the primary radical pair $P^+_{680}I^-$ in the range of 36–46 ns at 277 K [12–14] and 58 ns at 120 K [12]. For the primary charge separation step in the isolated reaction center, Schatz et al. [15] predicted a rate constant of 1/2.7 ps^{-1}, based on kinetic modelling of picosecond fluorescence and absorption data of PS II particles (≈ 60 antenna Chls). The first transient absorption experiments with subpicosecond time-resolution on the D_1-D_2-cyt-*b*-559 complex (in the presence of Triton X-100 under anaerobic conditions) by Wasielewski et al. [16] showed that at 277 K the excited primary donor P^*_{680} had a lifetime of 2.6 ± 0.6 ps, whereas the radical pair $P^+_{680}I^-$ was formed with a time constant of 3.0 ± 0.6 ps. This charge-separation process is accelerated as the temperature is lowered to 15 K to yield a P^*_{680} lifetime of 1.4 ± 0.2 ps [17]. By way of transient optical hole-burning at 4.2 K, Jankowiak et al. [18] revealed a lifetime of 1.9 ± 0.2 ps for P^*_{680} in the D_1-D_2-cyt-*b*-559 complex (in the presence of Triton X-100 and oxygen). Time-resolved fluorescence experiments revealed the lifetime of the radical pair $P^+_{680}I^-$ through its recombination fluorescence. Its relative quantum yield was found to depend on sample intactness [8,10] and temperature [19–21]. In addition to the radical pair lifetime, all these studies also reported a 5–7 ns lifetime component attributed to Chl which is energetically uncoupled from the primary photochemistry. In another study [22], the fluorescence decay data of the D_1-D_2-cyt-*b*-559 complex (in the presence of Triton X-100 under aerobic conditions) were analyzed as a continuous distribution of lifetimes rather than a finite sum of exponentials, as was done in all other studies cited above. It was shown that the lifetimes in the range longer than 6 ns mostly disappeared when the primary acceptor I was chemically pre-reduced [22].

Picosecond fluorescence components of the D_1-D_2-cyt-*b*-559 complex have been resolved and identified only partially so far. Mimuro et al. [19], reporting two additional lifetimes of 50 ps and 1.0–1.4 ns, suggested that the 50 ps component should reflect the primary charge separation. In the study by Crystall et al. [10], samples of higher quality were used, but due to the limited time resolution in these experiments, these authors did not comment on the two fastest components resolved, i.e., 1.5 ns and 0.1 ns. A 2–3 ps fluorescence lifetime component, reflecting the primary charge separation, as one would expect based on the subpicosecond absorption measurements [16,17] and hole-burning experiments [18], has not been reported so far. It was thus our aim to characterize the picosecond and nanosecond fluorescence decay kinetics of the stabilized D_1-D_2-cyt-*b*-559 PS II reaction center complex by single-photon-timing (SPT) fluorescence experiments with high time resolution. We report here the first results on an ultrafast fluorescence decay component that in our opinion reflects the primary charge separation and on energy transfer processes occurring in the reaction-center complex.

Materials and Methods

Samples

Unless stated otherwise, the D_1-D_2-cyt-*b*-559 complexes were prepared from spinach according to Refs. 1 and 9 with the following modifications. First, the loaded ion-exchange column was washed with 3–4 litres buffer (50 mM Tris-HCl (pH 7.2)/30 mM NaCl) containing 0.05% (w/v) Triton X-100 until the eluent was essentially colorless. Then Triton X-100 was exchanged by β-lauryl maltoside by washing the column with 200 ml of the same buffer containing 0.1% (w/v) β-lauryl maltoside. The reaction centers were eluted from the column with 200 mM NaCl in the same buffer with 0.1% (w/v) β-lauryl maltoside. The samples were stored at $-80\,^\circ$C without any further additions. Anaerobic conditions during SPT-experiments were established by addition of 0.1 mg/ml glucose oxidase, 5 mM glucose and 0.05 mg/ml catalase [10]. The preparations were spectroscopically characterized before usage by a Q_y-band absorption maximum at 674.5 ± 0.5 nm, a stationary emission maximum at 681.5 ± 0.5 nm (both values at 277 K) and a stationary emission maximum at 682.8 ± 0.5 nm at 77 K. To check for possible sample degradation during lifetime measurements, the stationary emission spectra were recorded in situ before and after the lifetime measurements, as these were shown to be sensitive to sample integrity [8,21]. The spectra and maxima were unaffected, indicating the absence of any significant sample degradation during lifetime experiments. For measurements at 277 K the samples were sealed in a cuvette with 1.5×1.5 mm cross-section (final Chl concentration of 5 μg Chl/ml). For low-temperature experiments a gas-cooled cryostat (Leybold VSK 4-300) was used. The samples were frozen quickly in a 0.1 mm cuvette (final Chl concentration of 50 μg Chl/ml) by immersion in liquid N_2

without any cryoprotector being added. Excitation light entered from the back face of the cuvette (almost 180° detection geometry).

Picosecond fluorescence decays

Picosecond fluorescence decays were measured with the single-photon-timing system described previously [23] upon excitation with laser pulses (620 nm, $\leq$ 15 ps FWHM, full width at half maximum) at a repetition rate of 4 MHz. As a detector, a microchannel plate photomultiplier (Hamamatsu MCP-R1564U-01) was used, in combination with a double monochromator (4 nm FWHM). This yielded a system response function of approx. 70 ps FWHM. The channel resolution of the time-to-amplitude converter (TAC) was either 5 ps or 41 ps. Decays were typically accumulated up to 25000 counts in the peak channel (in case of 5 ps/channel) or 50000 counts/peak channel (in case of 41 ps/channel). Decays measured at different detection wavelengths and/or with different time resolutions were analysed globally as a sum of exponentials. The fit quality was evaluated by its global χ^2 value and residual plots. The results are presented as decay-associated spectra (DAS) [23].

Results

Fluorescence decays at 277 K

For experiments at 277 K a fresh sample was taken for recording each single decay. Decays were recorded at emission wavelengths between 670–690 nm and with two different time resolutions (see Materials and Methods). All the 12 decays were globally analyzed as a sum of exponentials. Six lifetime components were needed to describe the whole data set adequately, as judged from the χ^2 values and the residual plots. The lifetimes found are: $\tau_1 = 2$ ps, $\tau_2 = 41$ ps $\pm$ 10%, $\tau_3 = 0.16$ ns $\pm$ 5%, $\tau_4 = 1.1$ ns $\pm$ 5%, $\tau_5 = 5.9$ ns $\pm$ 5% and $\tau_6 = 30$ ns $\pm$ 5%. The corresponding DAS are shown in Fig. 1. The fastest lifetime component, which was found close to 2 ps with a relative amplitude of at least 90% and a broad spectral band centered around 681 nm, is actually below the limit of the time resolution of the set-up used. For a lifetime component of approx. 2 ps we therefore have to accept a fairly large error. Thus the fastest component in our decay data may be in the range of $\tau_1 = 1$–6 ps. This error range has been estimated from extensive analyses of simulated decay data (see below). An ultrashort component is clearly needed in order to describe the decays accurately in the fast time region. For this reason we performed global analyses on the decays recorded with high time resolution only and fitted a time window of only 5 ns. In this case, five components are needed to describe the decays accurately, as judged from the χ^2 values and the plots of residuals, which are shown in Fig. 2. These lifetimes

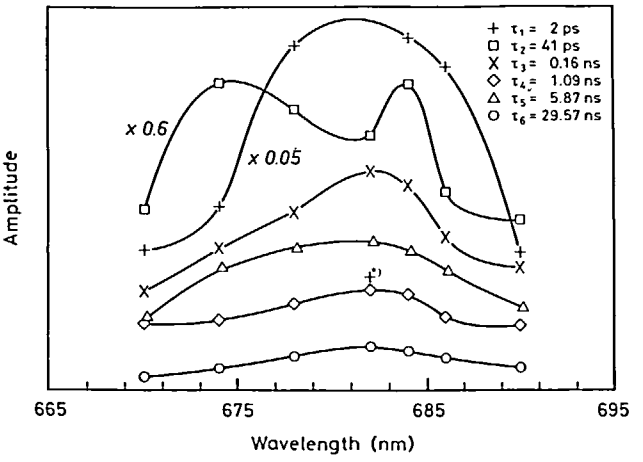

Fig. 1. DAS of the D_1-D_2-cyt-*b*-559 complex at 277 K, as calculated from the corrected amplitudes for the six lifetime components in the global analysis. 7 decays, recorded at different wavelengths with high time-resolution (5 ps/channel, 1000 channels) were combined with five decays, recorded at five different wavelengths, in the long time range (41 ps/channel, 1300 channels). * This data point was not taken into account for the interpolation of the DAS of τ_1. For the other lifetime components (τ_1–τ_6) this decay was taken into account.

are: $\tau_1 = 2$ ps, $\tau_2 = 42$ ps, $\tau_3 = 166$ ps $\tau_4 = 1.39$ ns and $\tau_5 = 10.6$ ns (Fig. 2B). When allowing for four components only, the fastest lifetime found was $\tau_1 = 29$ ps ($\tau_2 = 143$ ps, $\tau_3 = 1.33$ ns and $\tau_4 = 10.2$ ns). In this case, the residual plots show large deviations around time zero (Fig. 2A), indicating the necessity to include an ultrafast lifetime component. Also, the χ^2 values have increased significantly. The approx. 10 ns component in both analyses represents a mixture of the 6 ns and 30 ns components which cannot be resolved in this short fitting window.

In conclusion, these data indicate that the fastest fluorescence lifetime component in these D_1-D_2-cyt-*b*-559 complexes is in the range of 1–6 ps, has a relative amplitude of at least 90% (because of the limited time-resolution, its amplitude may actually be underestimated) and its DAS peaks around 681 nm. Both its DAS and its lifetime agree with what would be expected for the fluorescence of the primary donor P^*_{680}. We therefore attribute this ultrafast fluorescence component to the primary charge separation process, which has been shown by transient absorption measurements to occur with a lifetime of 3.0 ps [16].

The second lifetime component found (see Fig. 1) is $\tau_2 \approx 41$ ps. Its DAS shows two bands around 675 nm and 684 nm. It probably reflects an energy transfer process. This assignment will become clear only from comparison with the low-temperature data (see below). Two components ($\tau_3 = 0.16$ ns and $\tau_4 = 1.1$ ns) have their emission maximum around 682 nm. Their origin is probably also P^*_{680} emission, caused by still unknown mechanisms. The $\tau_5 = 5.9$ ns component has a DAS that is significantly broader than the other compo-

nents, with a blue-shifted contribution in addition to the peak around 682 nm. We interpret this component as being caused preferentially by Chl molecules that are energetically decoupled from the reaction center photochemistry as its lifetime is similar to that of isolated Chl in vitro [24]. The blue-shifted contribution could indicate that for some of these Chls the pigment–protein interaction is distorted. The longest lifetime found, $\tau_6 = 30$ ns, shows a maximum around 682 nm and is interpreted as radical pair recombination fluorescence, since none of the pigments contained in this complex is expected to have such a long excited-state lifetime. This fluorescence lifetime is somewhat shorter than the 36–38 ns reported by other groups [10,19–21], which might be due to the shorter fitting window applied here (54 ns vs. 90 ns). The relative yield of this recombination fluorescence at its emission maximum (682 nm) is 54%, as listed in Table I. This value is somewhat higher than the ones reported by other groups (44% [10], 48% [20,21]). Corrected for the contribution of uncoupled Chls ($\tau = 5.9$ ns), the relative quantum yield of this recombination fluorescence component is approx. 87% (Table I).

TABLE I

Lifetimes (τ) and relative quantum yields (ϕ) of the recombination fluorescence of several D_1-D_2-cyt-b-559 PS II reaction center complexes under stabilizing (i.e., β-lauryl maltoside and anaerobic) and non-stabilizing (i.e., Triton X-100 and aerobic) conditions

The relative yields given in parentheses (ϕ^c) are the values corrected for the contribution of uncoupled Chls to the total emission

Sample condition	277 K			77 K	
	τ (ns)	ϕ (ϕ^c)		τ (ns)	ϕ (ϕ^c)
Stabilized	30	54%	(87%)	35	14% (24%)
Stabilized [a]	38	48%	(80%)	41	9% (11%)
Not stabilized	–	–		31	10%
Not stabilized [b]	36	< 1% (< 40%)		40	9% (17%)

[a] From Ref. 21.
[b] From Ref. 19.

Fluorescence decays at 77 K

Fluorescence decays at 77 K were recorded in the wavelength range 666–690 nm, using a time-resolution of 5 ps/channel. We performed global analysis and five exponentials were needed to describe the data

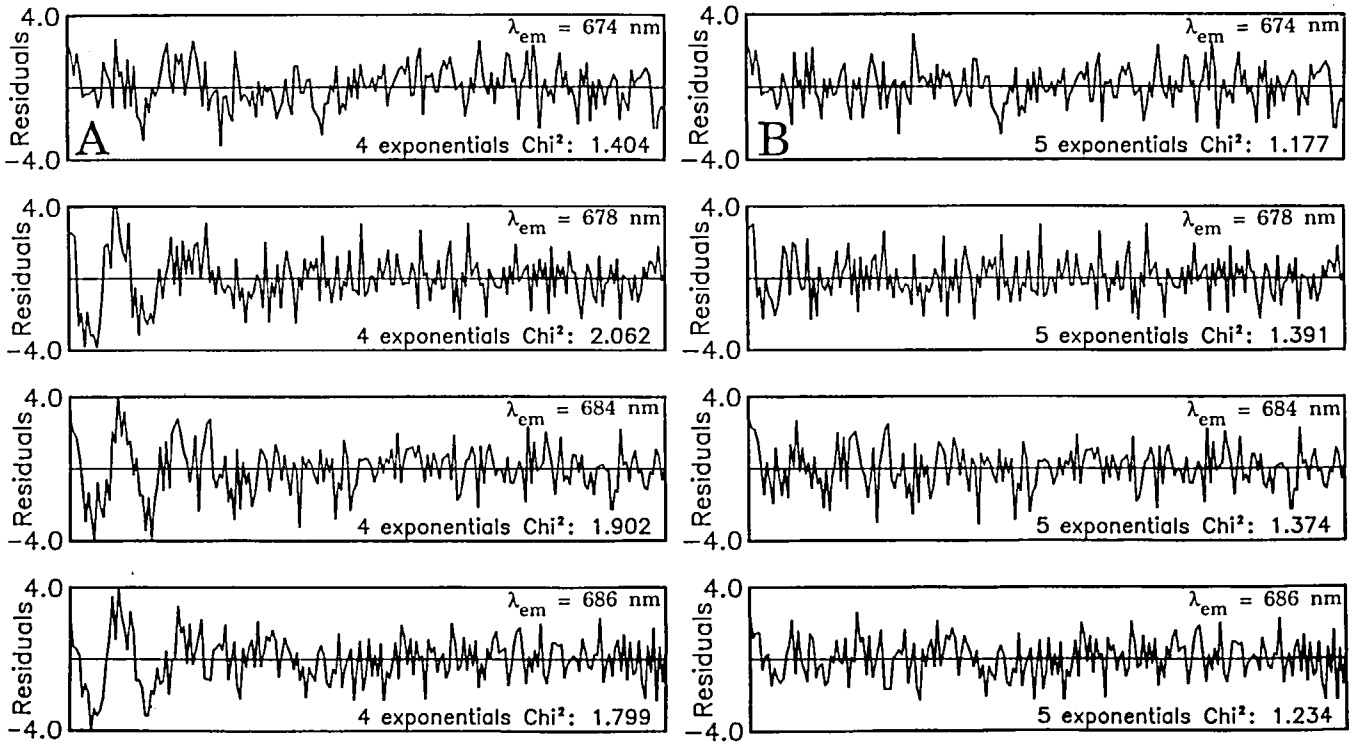

Fig. 2. Some typical weighted residual plots from the global analysis of the fluorescence decays from the D_1-D_2-cyt-*b*-559 complex at 277 K recorded with high time-resolution (5 ps/channel, 1000 channels). The χ^2 values and emission wavelengths are given for each decay. The residuals are shown for a four (A) and five (B) component fit for only the first 200 channels. The lifetimes found are for (A): $\tau_1 = 29$ ps, $\tau_2 = 143$ ps, $\tau_3 = 1.33$ ns and $\tau_4 = 10.2$ ns; for (B): $\tau_1 = 2$ ps, $\tau_2 = 42$ ps, $\tau_3 = 166$ ps, $\tau_4 = 1.39$ ns and $\tau_5 = 10.6$ ns. Note the systematic deviation from a random distribution in the case of the four component fit, particularly around zero time, which shows the presence of an ultrashort fluorescence lifetime component (see text).

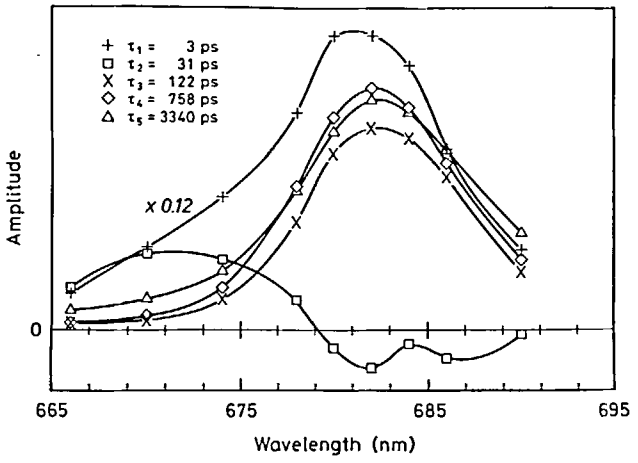

Fig. 3. DAs of the D_1-D_2-cyt-*b*-559 complex at 77 K, as calculated from the corrected amplitudes for the five lifetime components in the global analysis. Nine decays, recorded at nine different wavelengths, with high time-resolution (5 ps/channel, 500 channels) were analyzed.

over a window of 2.5 ns. The lifetimes found are: $\tau_1 = 3$ ps, $\tau_2 = 31$ ps $\pm$ 10%, $\tau_3 = 122$ ps $\pm$ 5%, $\tau_4 = 0.76$ ns $\pm$ 5% and $\tau_5 = 3.3$ ns $\pm$ 5%. The corresponding DAS are shown in Fig. 3. As at 277 K, a strong ultrafast fluorescence lifetime component ($\tau_1 \approx 3$ ps) with large relative amplitude is found with a broad DAS peaking around 680 nm. Again, a maximum error range of $\tau_1 \approx$ 1–6 ps has to be assumed (see below). This component can clearly not be ignored in the analysis, as we could verify by the same procedure as described above. We interpret this component, by analogy to the 277 K data, as reflecting the primary charge separation process. The DAS of the second lifetime component, $\tau_1 = 31$ ps, shows a positive band around 672 nm and a negative one around 683 nm. As a negative amplitude corresponds to a rise term in the fluorescence time profile, we interpret this component as an energy transfer process from a chromophore emitting around 672 nm to one emitting around 683 nm.

The lifetime components $\tau_3 = 122$ ps, $\tau_4 = 0.76$ ns and $\tau_5 = 3.3$ ns have their DAS maxima around 682 nm, with the spectrum of the latter component being somewhat broader than the other two. For the two longest-lived components (τ_4 and τ_5) it should be noted that their lifetimes might be underestimated because of the applied fitting window of only 2.5 ns. For this reason, we also performed experiments with a channel resolution of 41 ps/channel in the spectral range of 670–690 nm. Global analysis over a window of 54 ns revealed $\tau_4 = 4.9$ ns $\pm$ 5% and $\tau_5 = 35$ ns $\pm$ 5% (besides $\tau_1 < 0.1$ ns, $\tau_2 = 0.39$ ns and $\tau_3 = 1.89$ ns). The DAS of the 4.9 ns component (not shown) is somewhat broader, with an increased blue-shifted contribution, than those of the other components. This component is therefore attributed to uncoupled Chls, in analogy to the situation at 277 K. The longest lifetime found, 35 ns, has a DAS that again peaks around 682 nm. This component is thought to originate from radical-pair recombination. The radical-pair lifetime found here is again somewhat shorter than the values reported by other groups (40–41 ns in Refs. 19–21). The relative recombination fluorescence quantum yield is 14% (see Table I), which is again somewhat higher than the value of 9% reported in the literature for the same temperature [19–21].

It is interesting to report here also on the recombination fluorescence of D_1-D_2-cyt-*b*-559 complexes in the presence of Triton X-100 and oxygen. Under these non-stabilizing conditions the D_1-D_2-cyt-*b*-559 complex showed at 77 K a recombination fluorescence lifetime of 31 ns with a relative quantum yield of 10% (see Table I). This radical-pair lifetime is again somewhat shorter than reported in the literature [19] (see also above).

Simulated decays

In order to judge the experimental resolution limits for an ultrashort (1–6 ps) fluorescence lifetime component, we performed global lifetime analyses on simulated data sets. The experimentally found lifetimes and corresponding DAS of the high-time-resolution data (5 ps/channel) were simulated both for the situation at 277 K (i.e., 3.0 ps, 42 ps, 0.17 ns, 1.4 ns and 10.6 ns) and for 77 K (i.e., 1.4 ps, 31 ps, 0.12 ns, 0.76 ns and 3.3 ns). As the fastest lifetime found could not be determined accurately, we assumed that the primary charge separation takes place with 3.0 ps lifetime at 277 K [16] and with 1.4 ps lifetime at low temperature (1.4 ps was found at 15 K [17]). For both temperatures, the relative amplitude of such an ultrafast fluorescence component was systematically decreased from 90% (as found experimentally) down to 50%. The decay curves were convoluted with a generated system response function that had the experimental FWHM. Poissonian noise corresponding to the experimentally obtained counting statistics (typically 25 000 counts in the peak channel) was added to the decays. Such sets of simulated data (seven decays per spectrum) were then analyzed globally as a sum of four and five exponentials by the same procedure as applied to the experimental data. In Table II the results of these analyses are compiled. For the situation at 277 K, the ultrafast lifetime τ_1 can be recovered with an error increasing up to 50%, as the relative amplitude, A_1, is lowered to 50%. However, for relative A_1 values lower than 70%, leaving out this component no longer leads to significant deviations in the residual plots. This means that for amplitudes lower than 70%, such an ultrafast lifetime component could no longer be resolved under the experimental conditions used here. For higher amplitudes, however, the resolvability is clearly demonstrated by these re-

TABLE II

Results from global analyses on sets of simulated decay curves

The experimentally found lifetimes and corresponding DAS both for 277 K (3.0 ps, 42 ps, 0.17 ns, 1.39 ns and 10.6 ns) and 77 K (1.4 ps, 31 ps, 0.12 ns, 0.76 ns and 3.3 ns) were simulated. The relative amplitude, A_1, of the ultrafast fluorescence component, τ_1, was varied. The fit quality was assessed by its global χ^2 values and residual plots. If the data were fitted reducing the number of components by one, the fastest lifetime found was τ_1' and the quality of the fit was given by the $\chi^{2'}$ values. A qualitative comparison between the corresponding residual plots for the two different fits is also indicated, where a positive marker (+) means that leaving out the ultrafast component led to systematic deviations in the plots.

Simulated data		Global analyses					Residual plot distinguishable
τ_1 (ps)	A_1 (%)	τ_1 (ps)	A_1 (%)	χ^2	τ_1' (ps)	$\chi^{2'}$	
3.0	90	3.0	92	0.973	11	1.222	+
	80	3.1	83	0.947	20	1.169	+
	70	3.5	73	0.945	27	1.071	+
	60	3.9	62	0.943	31	1.010	−
	50	4.4	52	0.943	34	0.981	−
1.4	90	1.6	92	0.983	15	1.135	+
	80	2.1	79	0.984	25	1.031	−
	70	3.0	61	0.982	28	0.999	−
	60	3.3	52	0.981	29	0.991	−

sults. At low temperature (77 K), when the charge separation probably becomes faster (1.4 ps is assumed here), the corresponding resolution limit is shown to be 90% relative amplitude for the ultrafast fluorescence component.

If similar decays were simulated with the fastest component being 7 ps or longer, such a lifetime was recovered accurately (data not shown). On the other hand, if the fastest lifetime component became too short (below 1 ps), this component could not be recovered at all. These results on simulated data thus set a range of $\tau_1 = 1$–6 ps for the ultrafast fluorescence lifetime component resolved in this study.

Discussion

Charge separation and recombination processes

We have shown that the fluorescence decay kinetics of the stabilized D_1-D_2-cyt-*b*-559 PS II reaction center complex at 277 K as well as at 77 K are quite complex and require a description in terms of a sum of up to six exponential components with lifetimes between about 2 ps and 35 ns. The fastest component, with a lifetime of 1–6 ps, is proposed to reflect the primary charge separation process. It is obvious that the time resolution of the experiments presented here does not allow us to determine this fast lifetime very precisely. Nevertheless, the lifetime accuracy in combination with the DAS and the results of the analyses of simulated data (Table II) is good enough to make a proper assignment of this process to the primary charge separation step.

The radical-pair lifetime, reflected by its recombination fluorescence component, is $\tau \approx 30$ ns at 277 K and ≈ 35 ns at 77 K. The relative yield of this recombination fluorescence component is temperature-dependent: 87% at 277 K; 24% at 77 K (both values corrected for the emission of uncoupled Chls; see Table I). However it has to be emphasized here that, because of the absence of Q_A, the D_1-D_2-cyt-*b*-559 PS II reaction center complex does not show any variable fluorescence [25]. Therefore, these data do not allow conclusions to be drawn on the mechanistic origin of variable Chl fluorescence of PS II (in the presence of Q_A^-) as has been done elsewhere [22,25].

Free energy difference between the P^*_{680} and P^+I^- states

Having assigned the 1–6 ps component to the primary charge separation process and the 30 ns (277 K) and 35 ns (77 K) components to the radical-pair recombination process, we can apply a kinetic model to describe these two lifetime components. In a first approximation, we use a simple two-state model that describes an equilibrium between the excited state of the donor, P^*_{680} and the radical pair P^+I^-. This model is fully described by the four rate constants, k_a (radiationless plus radiative decay of P^*_{680}), k_{cs} (charge separation), k_{cr} (charge recombination back to P^*_{680}) and k_2 (charge recombination not leading to repopulation of P^*_{680}). According to this model, P^*_{680} exhibits biexponential fluorescence decay kinetics, one component reflecting the charge separation process, the other reflecting the recombination fluorescence. This kinetic model is fitted to the corresponding fluorescence components reflecting the charge separation and charge recombination processes. We assume that $k_a = 0.3$ ns^{-1}, similar to that used previously for PS II antenna Chl protein complexes [26]. However, as k_{cs} is faster than k_a by several orders of magnitude, the values found for the

other rate constants do not depend very much on the value assumed for k_a. Applying equilibrium thermodynamics, the free-energy difference between P_{680}^* and P^+I^- can be calculated according to Ref. 15 to be $\Delta G_{cs}^0 = -kT \cdot \ln(K_{cs}/k_{cr})$, where k is the Boltzmann constant and T the absolute temperature. For the situation at 277 K we thus calculate a ΔG_{cs}^0 of -124 meV ($k_a = 0.3$ ns^{-1}, $k_{cs} = 330$ ns^{-1}, $k_{cr} = 1.8$ ns^{-1} and $k_2 = 0.032$ ns^{-1}), whereas at 77 K a value of -46 meV results ($k_a = 0.3$ ns^{-1}, $k_{cs} = 330$ ns^{-1}, $k_{cr} = 0.29$ ns^{-1} and $k_2 = 0.028$ ns^{-1}). These values for the free-energy gap between excited state and radical pair are in reasonable agreement with those reported by Booth et al. [21]. Obviously, the free-energy gap is strongly temperature-dependent. The reason is unclear at present.

Energy transfer process

In addition to the ultrafast charge separation process we have resolved a lifetime component of 30–40 ps which indicates energy transfer from a pigment emitting around 672–674 nm to another one emitting around 683 nm. The identity of the two excited species involved in this energy transfer process is not entirely clear at present. The donor pigment could either be accessory Chl [11,18,27] or pheophytin [18,27,28], whereas for the energy-accepting species both P_{680} and pheophytin are possible candidates [11].

The energy-transfer component was resolved well only at a temperature of 77 K (Fig. 3) and below (data not shown). At 277 K, the DAS corresponding to this lifetime component shows a strong positive amplitude in the spectral region of the energy acceptor. This probably indicates that this lifetime component actually is a sum of two (or even more) processes occurring with similar kinetics. One of these processes would be an energy transfer as discussed above, the other a process of still unknown identity with its DAS peaking around 682 nm. The relative contribution of these processes to the overall DAS could very well depend on temperature. In the light of these as yet incompletely resolved kinetics, it could very well be that actually more than two pigment species are involved in energy transfer processes in the reaction center. This has recently been proposed, based on hole-burning experiments, by Tang et al. [27,29], who reported an excited state lifetime for accessory Chl and pheophytin *a* of 12 ps and 50 ps, respectively, at 1.6 K. The 30–40 ps lifetime component reported here could thus be a composition of these different processes. Further low-temperature fluorescence kinetics experiments indeed indicate the involvement in energy-transfer processes of at least three spectrally different species (Roelofs et al., unpublished data).

It has to be noted, however, that these energy-transfer kinetics are unexpectedly slow. In the reaction-center complex of purple bacteria the excited-state lifetimes for the accessory pigments (bacteriochlorophylls and bacteriopheophytins) were found to be no greater than 100 fs [30]. The slowing-down of this energy transfer in the reaction center of PS II could indicate that the arrangement of the pigments involved is strongly distorted in these complexes, perhaps due to the isolation procedure. However, as in these preparations the charge-separation process in the majority of the reaction centers still occurs with ultrafast kinetics ($\tau \approx 1$–6 ps with a relative amplitude of at least 90%), a substantial distortion of the reaction center geometry is quite unlikely. Relative to the amplitude of the fluorescence component reflecting the primary charge separation ($\tau = 1$–6 ps), the amplitude of the energy donor fluorescence is only 7% (at 277 K, Fig. 1) or 3% (at 77 K, Fig. 3). Although these data do not allow for complete resolution of the energy transfer processes in the reaction center (see above), in a rough approximation it indicates that these lifetime components originate from only a small fraction of excited energy-donating pigments. At the moment it remains unclear whether this decelerated energy-transfer process occurs in a fairly small fraction of (damaged) reaction centers, or whether the energy donating pigments were excited with a small probability (because of a small partial absorbance at the excitation wavelength used in this study). Alternatively, it could be concluded that femtosecond energy-transfer kinetics among and/or from the accessory pigments in the PS II reaction center is perhaps not a functional necessity for charge separation within a few picoseconds

We also note here that Govindjee and co-workers [22] already proposed the occurrence of picosecond energy transfer processes in the D_1-D_2-cyt-*b*-559 complex, since blocking the primary charge separation by prereduction of pheophytin did not lead to a complete disappearance of the picosecond lifetime contributions. However, in that study the energy transfer process was not shown unequivocally, since no rise terms were observed. Also, a fast quenching of the excited states in the reaction center is very likely to occur under the conditions applied in that study, since reduced pheophytin *a* is an efficient quencher [25,31].

Apart from the charge separation, energy transfer and charge recombination processes, three additional components have been resolved, one of them ($\tau \approx 5$–6 ns) being attributed to Chls which are uncoupled from the reaction-center photochemistry. The two other lifetime components found ($\tau \approx 120$–170 ps and ≈ 1–2 ns) probably originate from P_{680}^* (maxima of DAS around 682 nm). A possible mechanistic origin could also be recombination fluorescence from radical-pair states. Any assignment of these two components remains quite speculative at present, however, and further studies are required to arrive at an accurate

kinetic model for the primary photophysics and photochemistry of the isolated reaction center of PS II.

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