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Primary processes and structure of the Photosystem II reaction center: II. Low-temperature picosecond fluorescence kinetics of a D_1-D_2-cyt-*b*-559 reaction center complex isolated by short Triton exposure

Theo A. Roelofs [a,1], Stefan L.S. Kwa [b], Rienk van Grondelle [b], Jan P. Dekker [b] and Alfred R. Holzwarth [a,1]

[a] *Max-Planck-Institut für Strahlenchemie, Mülheim an der Ruhr (Germany)* and [b] *Department of Biophysics, Physics Laboratory of the Free University, Amsterdam (The Netherlands)*

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The fluorescence kinetics of a D_1-D_2-cyt-*b*-559 reaction center complex isolated by short Triton-exposure has been measured with picosecond resolution as a function of temperature below 150 K. The data were analyzed by combined global analysis of data measured with different time resolutions and are presented as decay-associated fluorescence spectra (DAS). Emphasis is given to the resolution and assignment of the fast components below 100 ps. Six lifetime components were generally necessary for a good fit of the data over a long time-range. The shortest lifetime of 13–24 ps (depending on temperature) is attributed to an energy transfer from (accessory) Chlorophyll to P680, presumably via pheophytin. A component in the range of 40–70 ps is attributed to energy transfer from pheophytin to P680. These components can only be properly resolved and assigned from measurements at temperatures below about 40 K. An ultrashort component of 1–6 ps, which had been resolved in our previous measurements (Biochim. Biophys. Acta (1991) 1060, 237–244) was not resolved in the particles studied here. We propose that the absence of the ultrashort component in this study is attributable to the larger chlorophyll content of the present short-term Triton-exposed D_1-D_2-cyt-*b*-559 preparation as compared to the previous long-term Triton-exposed RC particles. In the particles studied here the *effective charge separation kinetics* in the short time-range is rate-limited by relatively slow energy transfer components (lifetimes from 13 to 24 ps) upon preferential excitation of the accessory chlorophylls. In the long time-range a multicomponent charge recombination kinetics giving rise to four fluorescence lifetimes in the range of 1.56–49.7 ns are resolved. All these components show basically identical DAS with maxima near 682 nm.

Introduction

Since the first report on the purification and identification of the D_1-D_2-cyt-*b*-559 reaction center complex of Photosystem II (PS II) from higher plants [1], research on the PS II primary photochemistry has been intensified. Several papers have indicated that the lifetime of the P680 excited singlet state in isolated reaction centers is a few picoseconds, as revealed by transient absorption measurements (3 ps at 277 K [2], 1.4 ps at 15 K [3]), fluorescence kinetics experiments (1–6 ps at 277 K and 77 K [4]), and transient hole-burning studies (1.9 ps at 1–4 K [5–7]). These kinetics are in agreement with earlier predictions of Schatz et al. [8], who calculated by extrapolation a charge separation lifetime of about 3 ps from P680 after modelling picosecond fluorescence and absorption data on PS II core particles. Recently, results from femtosecond transient absorption spectroscopy have indicated two lifetimes of about 400 fs and 3.5 ps of unknown origin [9]. Whether or not P680* directly generates the primary radical pair $P680^+Pheo^-$ remains to be clarified. A 21 ps lifetime was reported for pheophytin reduction [9], in contrast to the 3 ps lifetime assigned to this

Correspondence to: A.R. Holzwarth, Max-Planck-Institut für Strahlenchemie, Stifstr. 34–36, D-4330 Mülheim an der Ruhr, Germany.
[1] Present address: Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, 1 Cyclotron Road, Berkeley, CA 94720 USA.
Abbreviations: SPT, single-photon timing; PS II, Photosystem II; BChl, bacteriochlorophyll; EPR, electron paramagnetic resonance; FWHM, full width at half maximum; Pheo, pheophytin; RC, reaction center; P680, primary electron donor of RC II; β-Car, β-carotene; Chl, chlorophyll; DAS, decay-associated spectrum.

process in Ref. 2. The radical pair $P680^+Pheo^-$ then decays in the tens of nanoseconds time domain [4,10–13] in a multiexponential fashion with at least two lifetimes [14,15].

A number of recent results suggest that PS II reaction centers contain a pair of Chl *a* molecules analogous to the special pair of BChl *a* molecules in reaction centers of purple bacteria [16]. Preferential excitation of P680 resulted in an about 2-times higher initial absorbance change than when accessory chlorophylls were excited [9]. Also, polarized fluorescence excitation spectra (in which the value of the limiting anisotropy suggested a dimeric arrangement with angles of Q_y transitions similar to those in the bacterial reaction center [17]) and absorbance difference spectra of the triplet state (showing a small positive feature near 671 nm attributed to one of the monomer bands of P680 [18]) are explained most easily by assuming a dimeric arrangement. Electron spin resonance measurements of the triplet state, however, suggest that the triplet state is localized on a monomeric Chl *a* molecule with an orientation similar to that of the accessory BChl molecules in the bacterial reaction center [19].

There is substantial evidence that the PS II reaction center contains two Pheo *a* molecules with orientations very similar to the corresponding BPheo molecules of the bacterial reaction center [17,20]. The number of accessory Chl and β-carotene molecules, however, is different for both systems. Several recent reports point to a 6 : 2 : 2 stoichiometry of Chl : Pheo : β-Car in many recently isolated D_1-D_2-cyt-*b*-559 complexes [21–24]. The average orientation of the accessory Chl molecules absorbing near 670 nm differs significantly from the orientation of the accessory BChl molecules in the bacterial reaction center [17]. The number of accessory Chl molecules in the PS II reaction center, however, may depend on the detailed procedure by which the reaction center complexes have been isolated. It was shown that prolonged exposure to Triton X-100 leads to the loss of pigment [25], most likely 2 Chl *a*nd 1 β-Car molecule [24]. This suggests that reaction centers isolated after prolonged (overnight) Triton X-100 exposure contain less Chl than complexes isolated under less extensive Triton treatment. It is not clear at present what influence the number of (accessory) Chl molecules might have on the primary photochemistry in these particles. Comparative studies of different preparations are thus appropriate.

In a previous study, we presented the picosecond and nanosecond fluorescence kinetics of reaction center particles that have been subjected to prolonged exposure (overnight washing) to Triton X-100 basically following the original protocol by Nanba and Satoh [1] but subsequently stabilized in β-lauryl maltoside [4]. These particles contained on average 4 Chl per 2 Pheo as shown by detailed pigment analysis [26]. An ultrashort component, with a lifetime of 1–6 ps, and a dominant relative amplitude was resolved and attributed to the primary charge separation process. (Note that throughout this paper we will refer to the 1–6 ps component as 'ultrashort'.) Furthermore, indications for picosecond energy transfer processes (with 30–40 ps kinetics) within the reaction center were resolved at low temperature in addition to several long lifetimes in the nanosecond range.

In this study we report on the low temperature fluorescence kinetics of reaction center particles that have only been exposed shortly to Triton X-100 [17]. Particles prepared under similar conditions were shown to contain 6 Chl *a* and 2 Pheo *a* molecules [23]. Thus, the particles used in the present study contained more accessory Chl than the particles used in our previous study which had been isolated under prolonged Triton exposure [4] and contained only 4 Chl/2 Pheo [26]. Our main aim for this work consisted in the characterization of the picosecond fluorescence lifetime components reflecting the charge separation and the energy transfer processes. The intrinsic charge separation step could not be resolved, however, for reasons discussed in detail.

Materials and Methods

Isolation of the RC complexes

Photosystem II reaction center (D_1-D_2-cyt-*b*-559) complexes were isolated by means of a short (2 × 20 min) Triton X-100 treatment of spinach CP47-RC preparations [25] as described by Kwa et al. [17]. The samples were stored at −70°C in BTT (20 mM BisTris

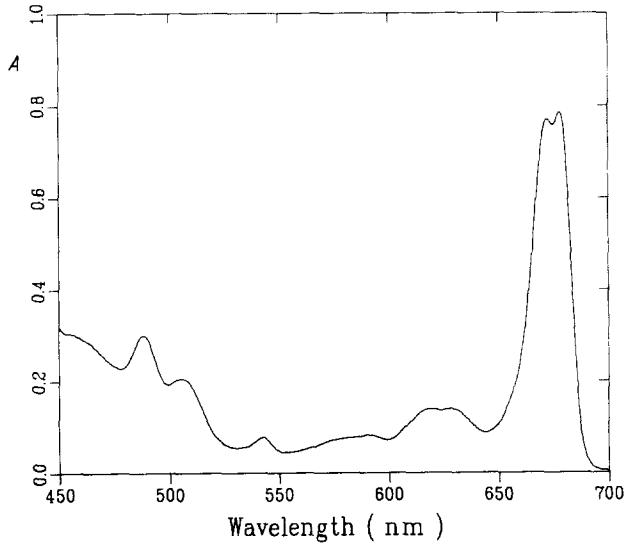


Fig. 1. Absorption spectrum at 77 K of the D_1-D_2 preparation used in this study. The spectrum is typical of a preparation with 6 Chl/2 Pheo.

(pH 6.5), 20 mM NaCl, 10 mM $MgCl_2$ and 1.5% taurine) with 100 mM $MgSO_4$ and 0.03% β-lauryl maltoside until further use. The quality of the samples was checked by 77 K absorption, fluorescence and excitation spectra [17]. The particles were characterized by a room temperature absorption maximum at 675.5 nm and by the absence of a shoulder or peak at 672 in the 77 K fluorescence emission spectrum [17] which suggested that unconnected pigments did not contribute to the steady-state emission. For the fluorescence kinetics measurements, the samples were diluted to approx. 40 μg Chl/ml with BTT + 0.03% lauryl maltoside. Anaerobic conditions were established by incubation with 0.1 mg/ml glucose oxidase, 5 mM glucose and 0.05 mg/ml catalase [27]. The samples were filled into a 0.1 mm pathlength cuvette and shock-frozen by immersion into liquid nitrogen without any cryoprotector being added. The sample was subsequently transferred into a gas-cooled cryostat (Leybold VSK 4-300). A back-face excitation geometry was used. A 77 K absorption spectrum of this preparation is shown in Fig. 1. This spectrum is typical of D_1-D_2 preparations with 6 Chl/2 Pheo.

Fluorescence kinetics

Picosecond fluorescence decays were measured with the single-photon-timing (SPT) system described previously [28] upon excitation with laser pulses of 620 nm or 636 nm (full width at half maximum $\leq$ 15 ps) at a repetition rate of 800 kHz with ≤ 4 mW/mm^2. A microchannel plate photomultiplier (Hamamatsu MCP-R1564U-01) was used as a detector, in combination with a double monochromator (4 nm fwhm). This yielded a system response function of approx. 50 ps fwhm, i.e., somewhat narrower than in our previous measurements [4]. The channel resolution of the time-to-amplitude converter (TAC) in connection with the analog-to-digital converter was varied from 2 ps up to 41 ps in order to cover a large dynamic range. Decays were typically accumulated to 20000–30000 counts in the peak channel (high time resolution) or 60000 counts/peak channel (low time resolution). Decays measured at different detection wavelengths in the range 666–691 nm and/or with different time resolutions were combined in one global lifetime analysis. In this global analysis procedure the fluorescence decays, $F(t, \lambda_{em})$ were fitted with a sum of exponentials ac-

TABLE I

Lifetimes and amplitudes of the fluorescence components resolved by combined global data analysis of fluorescence decays measured at several emission wavelengths and at several time resolutions

Temperature (K)	Assignment:	Energy transfer	Energy transfer	Partial charge recombination	Charge recombination	Charge recombination	Charge recombination	Comment
150	lifetimes (ns)	0.022	0.067	0.343	2.28	6.42	37.6	
	amplitude (%) [a]	22	23	19	20	14	1.4	
150	lifetimes (ns)	–	–	2.27	6.52	38.1	–	three-component fit; $\chi^2 = 1.30$; fitting window 2 to 76 ns
	lifetimes (ns)	–	–	1.56	4.40	11.2	49.7	four-component fit; $\chi^2 = 1.17$ fitting window from 2 to 76 ns
	amplitude (%) [a]	–	–	42	46	9.2	2.3	
77i	lifetimes (ns)	0.024	0.128	0.839	2.76	5.7	39.5	
	amplitude (%) [a]	20	27	17	23	12	0.7	
	lifetimes (ns) [b]	0.031	0.122	0.758	–	3.34		
	amplitude [c]	7.3	1.5	2.2	–	4.0		
	amplitude [d]	60.0	5.1	2.7	–	4.0		
20	lifetimes (ns)	0.018	0.066	0.54	3.9	–	–	fitting window from 0 to 1 ns
	amplitude (at 683 nm)	−3.4	3.8	4.2	9.5			

[a] Relative amplitudes at 683 nm.
[b] Lifetimes at 77 K in previous study (Ref. 4).
[c] Normalized amplitudes at 672 nm at 77 K in previous study (Ref. 4).
[d] Normalized amplitudes at 672 nm at 77 K in this study. Note that the components 1 and 2 assigned to energy transfer are significantly higher than in the previous study with long-term Triton-exposed RC particles (Ref. 4).

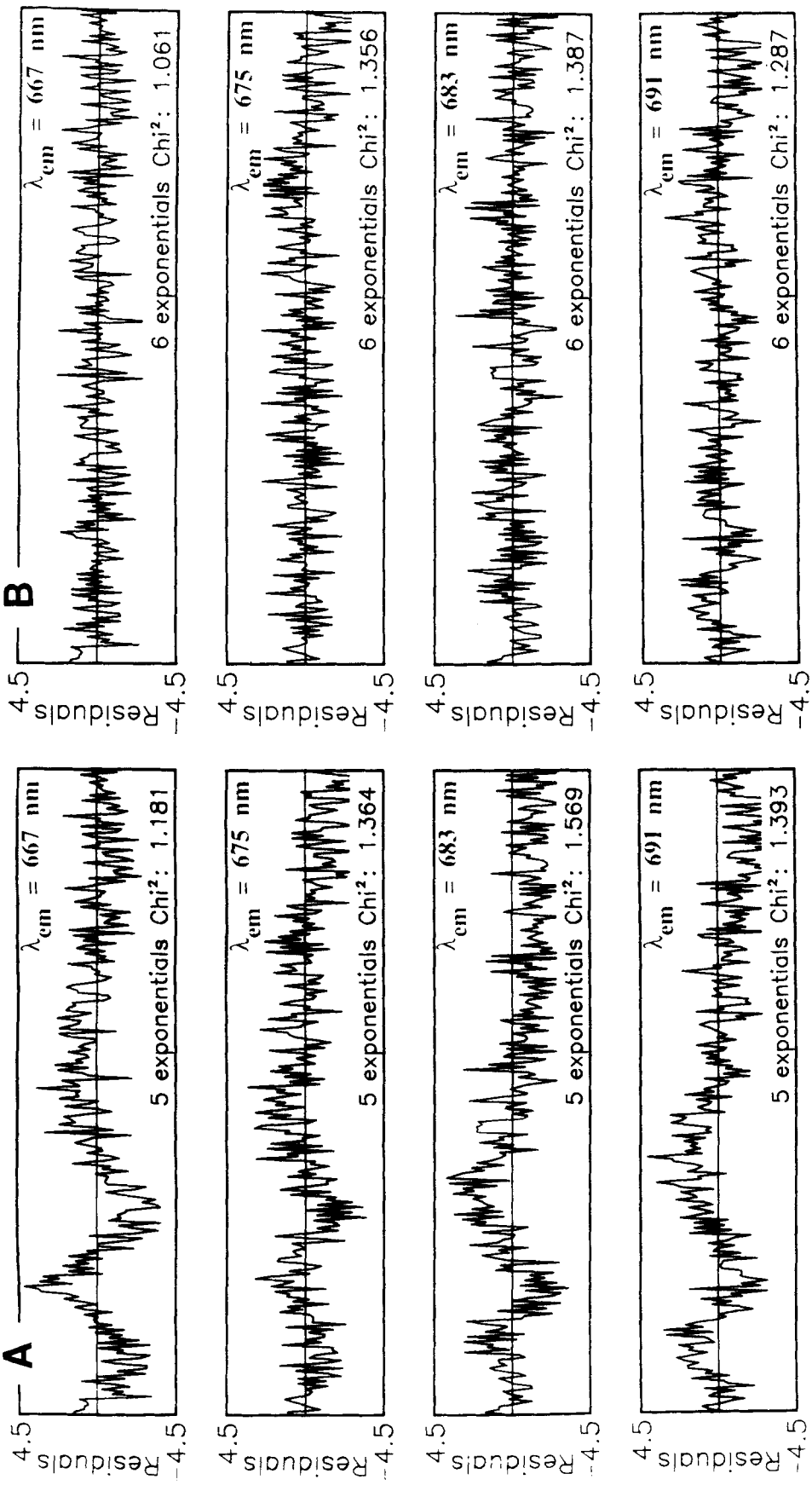


Fig. 2. Typical weighted residuals plots of the combined global lifetime analysis of the fluorescence kinetics of RC particles at 150 K (upon excitation at 636 nm), allowing for 5 (left) and 6 (right) components. The channel resolution was 2 ps; the plots show the first 250 channels only.

cording to Ref. 28:

$$F(t, \lambda_{em}) = \sum_{i=1}^{n} a_i(\lambda_{em}) \cdot \exp(-t/\tau_i) \quad (1)$$

The fit quality was assessed by its reduced χ^2 value, plots of the weighted residuals and corresponding autocorrelation functions. The results are presented as decay-associated spectra (DAS) which are plots of the amplitudes a_i for the lifetime component τ_i versus the emission wavelength λ_{em}.

Results

Temperature 150 K

The fluorescence decay kinetics at 150 K of the shortly Triton-exposed RC preparation upon excitation at 636 nm could be described by a sum of 6 exponential lifetime components. The lifetimes and relative amplitudes at 683 nm of these components were $\tau_1 = 22$ ps (22%), $\tau_2 = 67$ ps (23%), $\tau_3 = 340$ ps (19%), $\tau_4 = 2.28$ ns (20%), $\tau_5 = 6.42$ ns (14%) and $\tau_6 = 37.6$ ns (1.4%). This resolution resulted from a combined global lifetime analysis in which 30 decays recorded at 7 different wavelengths (between 667 nm and 691 nm) with 3 different time resolutions (18 decays with 2 ps/channel, 7 decays with 10 ps/channel and 5 decays with 41 ps/channel) were analysed together, each over a range of about 2000 channels. The reduced χ^2 value for this fit was 1.22. The weighted residuals for decays with 2 ps channel resolution are plotted in Fig. 2A for the first 500 ps (see Table I for a collection of all lifetime and amplitude data). If only five lifetime components were allowed for, the two shortest components mixed to a single component of 35 ps lifetime, while the other components were only slightly changed (274 ps, 2.12 ns, 6.18 ns and 36.7 ns). The reduced χ^2 value for this 5-component fit was 1.30 and the weighted residuals for decays with 2 ps channel resolution for the first 500 ps, are also plotted in Fig. 2B. These residuals plots show strong deviations from a random distribution, especially in the short time-range, which disappear when allowing for a sixth component (Fig. 2A).

The DAS of the 6 components found at 150 K are shown in Fig. 3. The shortest lifetime, 22 ps, has a DAS that peaks around 671 nm, with a shoulder around 677 nm, and has a dominant amplitude over the whole spectral range. Its blue-shifted maximum and its short

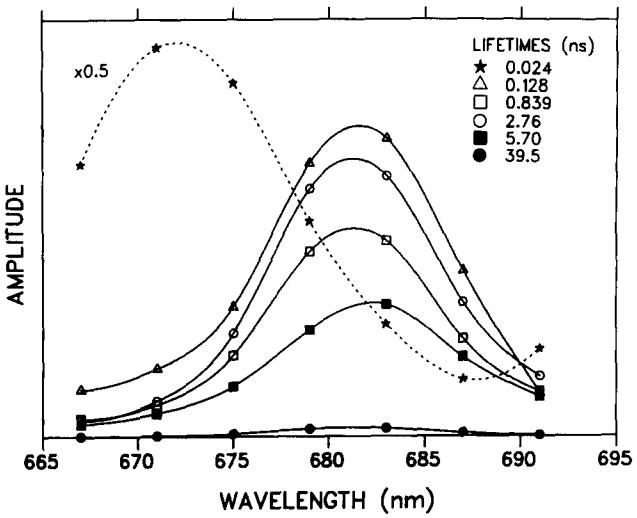


Fig. 4. DAS of RC particles at 77 K ($\lambda_{exc} = 620$ nm), as calculated from the corrected amplitudes of the lifetime components in the combined global lifetime analysis. The curves between the data points are interpolated with third-order polynomials.

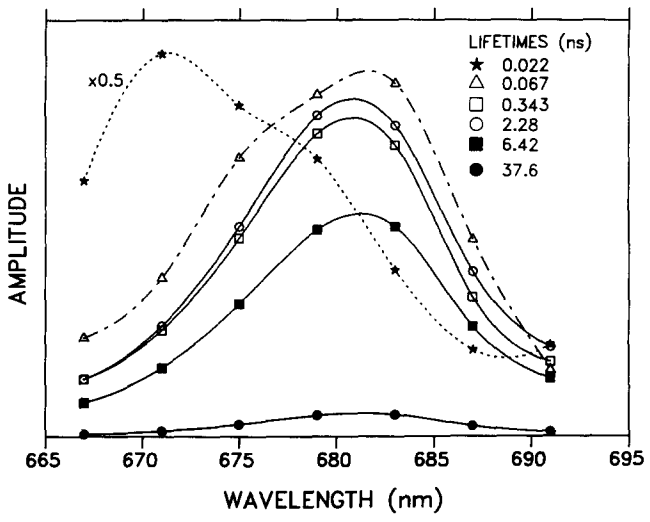


Fig. 3. DAS of RC particles at 150 K ($\lambda_{exc} = 636$ nm), as calculated from the corrected amplitudes of the lifetime components in the combined global lifetime analysis. The curves between the data points are interpolated with third-order polynomials.

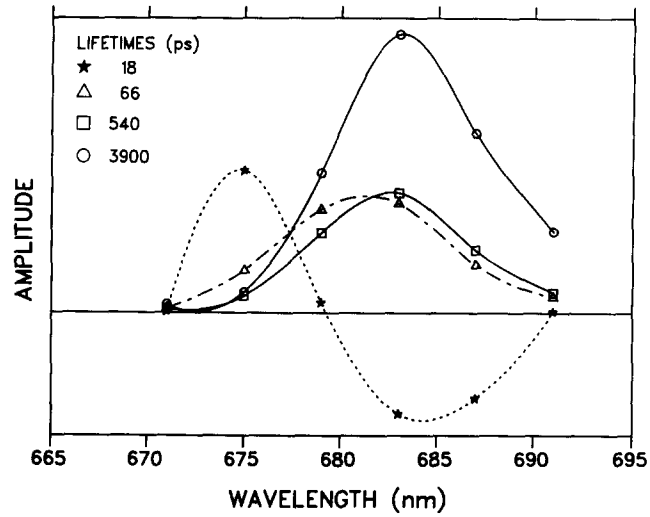


Fig. 5. DAS of RC particles ($\lambda_{exc} = 636$ nm) at 20 K, as calculated from the global lifetime analyses (fitting window from 0 to 1 ns) of decays recorded with a resolution of 4 ps/channel. The curves between the data points are interpolated with third-order polynomials.

lifetime suggest that this component originates from an accessory Chl [18], transferring its excitation energy to another pigment. The $\tau_1 = 22$ ps component resolved here has been attributed to energy transfer previously based on the resolved rise term (negative amplitude) at long wavelengths [4]. In this study, the energy transfer character of this component can be shown convincingly only at still lower temperatures (see below). The DAS of the τ_2-component (67 ps) has its maximum around 682 nm, with a shoulder around 677 nm. The main part of this DAS can probably be assigned to P680*. However, the blue-shifted contribution in its DAS indicates that it may contain contributions from a species with an emission spectrum slightly blue-shifted relative to that of P680. The other four lifetime components all have nearly identical DAS with their maxima around 681 nm, suggesting P680 as the emitting chromophore. The longest lifetime found, 38 ns, is mechanistically

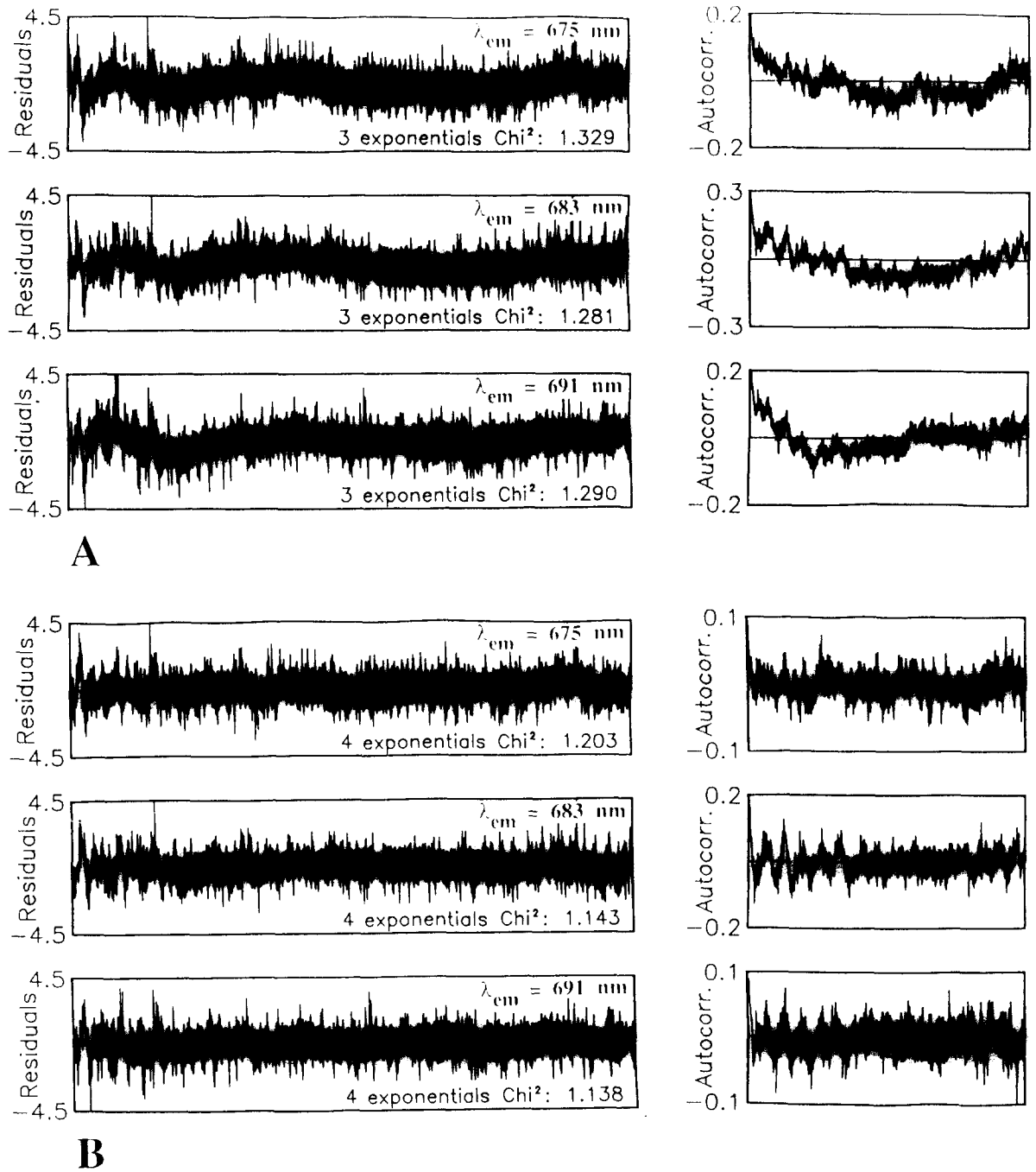


Fig. 6. Weighted residuals plots and corresponding autocorrelation functions for a three- (A) and four- (B) component global lifetime analysis of the fluorescence kinetics of RC particles at 150 K, upon excitation at 636 nm. The channel resolution was 41 ps/channel, the fitting window ranged from 2 to 76 ns.

attributed to the radical pair recombination process leading to a repopulation of P680*, since none of the chromophores present in the RC is expected to have such a long excited state lifetime. The relative quantum yield of this component is approx. 27% at 150 K.

Temperature 77 K

In order to allow for an optimal comparison between the fluorescence kinetics of the two different RC preparations, we also performed measurements under conditions identical to the ones reported in the previous study [4], i.e., at 77 K and excitation at 620 nm. In a combined global lifetime analysis 20 decays, recorded at three different time resolutions (six decays with 2 ps/channel, 7 decays with 10 ps/channel and 7 decays with 41 ps/channel), were simultaneously fitted over a window of 2000, 1200 and 1200 channels, respectively. A sum of six exponential components was needed to get an accurate fit, with a reduced χ^2 value of 1.22. These lifetimes and relative amplitudes at 683 nm were $\tau_1 = 24$ ps (20%), $\tau_2 = 128$ ps (27%), $\tau_3 = 0.84$ ns (17%), $\tau_4 = 2.76$ ns (23%), $\tau_5 = 5.70$ ns (12%) and $\tau_6 = 39.5$ ns (0.7%). The corresponding DAS are shown in Fig. 4. The τ_1 component (24 ps) shows a DAS that peaks around 672 nm and is the dominant component. All other components have similar DAS, peaking around 682 nm. The relative quantum yield of the ≈ 40 ns recombination fluorescence component is 15%.

Temperatures below 32 K

In order to obtain more information on the energy transfer processes, we recorded fluorescence decay kinetics (upon excitation at 636 nm) with a resolution of 4 ps/channel, at very low temperatures in the range of 15 K–32 K. Fitting the decays globally over the first nanosecond, four lifetime components were needed to give a good fit quality. Lifetimes of 18 ps, 66 ps, 0.54 ns and 3.9 ns were fitted to the kinetics at 20 K. The DAS of the 18 ps component (Fig. 5) exhibits a strong positive band around 675 nm and a strong negative band around 684 nm. The DAS of the 66 ps component shows only positive amplitudes with a maximum around 681 nm. The DAS of the two longest-lived components have their maxima at 683 nm, i.e., slightly red-shifted, as compared to the 66 ps lifetime component. The measurements at 15 K and 32 K gave qualitatively similar results with fastest lifetimes of 13 ps at 15 K and 18 ps at 32 K.

Lifetimes of the radical pair states

In the combined global lifetime analysis (Figs. 3 and 4) a single exponential component was found with a lifetime significantly longer than 6 ns. This component has been identified as radical pair recombination fluorescence (vide supra). However, inspection of the residuals plots in the long time-range using an extended 76 ns fitting window indicates that this long-lived exponential is not sufficient to give an accurate fit. For this reason we performed a global lifetime analysis of the decays recorded at 150 K with a time resolution of 41 ps/channel only. In order to put more weight on the long nanosecond kinetics, the first part of the decays was left out, i.e., the decays were fitted over a time-range from 2 to 76 ns. Fig. 6 shows the plots of the weighted residuals and the autocorrelation of the residuals for a 3- (A) and 4- (B) component fit. Allowing for three components only, the resulting lifetimes (and relative amplitudes at 683 nm – in parentheses) are 2.27 ns (59%), 6.52 ns (37%) and 38.1 ns (3.8%). The reduced χ^2 value is 1.30. These results are comparable to those found in the combined global lifetime analysis shown in Fig. 3 (taking into account the different fitting window). The plots of the weighted residuals (Fig. 6A), show, however, long-term deviations from a random distribution which show up even more pronouncedly in the corresponding autocorrelation functions. Fitting the decays with 4 components resulted in lifetimes of 1.56 ns (42%), 4.40 ns (46%), 11.2 ns (9.2%) and 49.7 ns (2.3%). The reduced χ^2 value for this fit improved to 1.17 and the residuals and autocorrelation functions (Fig. 6B) are free from deviations in the long time-range. From the corresponding DAS, shown in Fig. 7, it can be seen that all four components have basically identical DAS with maxima near 682 nm.

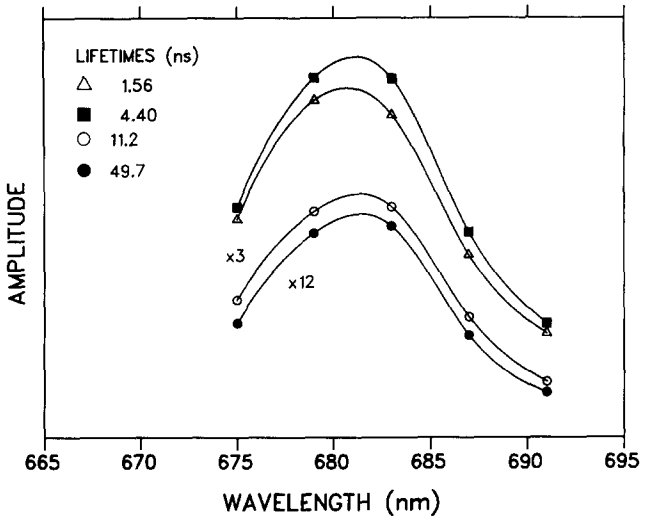


Fig. 7. DAS of RC particles at 150 K ($\lambda_{exc} = 636$ nm), as calculated from the global lifetime analysis in which the decays were fitted over a time window from 2 to 76 ns. The channel resolution was 41 ps/channel. The curves between the data points are interpolated with third-order polynomials.

Discussion

Primary charge separation process

In the previously studied RC preparation that had been extensively exposed to Triton X-100 during the

preparation which resulted in a Chl/Pheo ratio of 2:1, a 1–6 ps lifetime component with dominant amplitude ($\geq 90\%$) and a DAS peaking around 680 nm was resolved and attributed to the primary charge separation process [4]. In the nanosecond time-range the kinetics of the particles studied here are similar to those found in the previous study. However, in the short time range there are striking differences: (i) an ultrashort lifetime component of a few picoseconds is not resolved in the RCs studied here, (ii) the τ_1 component reported here (Figs. 3 and 4) shows a positive amplitude around 672 nm that is drastically higher (by a factor of about 8) as compared to the corresponding amplitude in the longer-exposed RCs (cf. Table I for a comparison), and (iii) it lacks the rise term around 682 nm, present in the longer Triton-exposed RCs [4]. These differences will be now discussed in relation to the different pigment contents in these two types of RC particles.

Fig. 2B shows that the 6-component fit applied here, with the shortest lifetime being 22 ps, describes the decays very well during the first 500 ps and no additional ultrafast component is required for a good fit. Despite the lack of the ultrashort charge separation component in the RC particles studied here, the relative recombination fluorescence quantum yield of 15% at 77 K (vide supra) is very similar to the corresponding quantum yield of 14% found previously at 77 K [4]. This implies that the charge separation process should occur with a similar efficiency in both types of RC particle. This, in turn, suggests that the primary charge separation process actually takes place within a few picoseconds also in the particles studied here. Apparently, the ultrashort fluorescence component reflecting the primary charge separation process is beyond the resolution of the SPT set-up under the conditions of these measurements despite the somewhat narrower time-response as compared to our previous study [4]. We suggest that this difference in the kinetics of the two RC particles is caused by their different pigment contents which has substantial influence on the observable kinetics.

Based on the analyses of simulated decay kinetics, some limits for the resolution of an ultrashort component (with a lifetime of a few ps) in our SPT experiments were estimated and described in detail previously [4]. Using the same procedure to estimate the resolution for the experimental conditions used in this study, i.e., a system response function with a fwhm of 50 ps, a channel resolution of 2 ps/channel and a signal-to-noise ratio corresponding to about 30000 counts in the peak channel (see Materials and Methods), the following limiting values resulted: a lifetime component of 2 ps would not be resolved if its relative amplitude was less than 65%. For a 1 ps lifetime component the corresponding amplitude limit is 80%. This shows that if for some reason the charge separation component is sufficiently reduced in its amplitude, this fluorescence component would not be resolved experimentally.

Reduction of the relative amplitude of the ultrashort fluorescence component in the short-time Triton-exposed RC particles studied here, as compared to the situation in the longer-exposed particles [4], can be envisaged by taking into account the different pigment composition of these two RC preparations. A positive amplitude for the ultrafast charge separation component is only expected when P680 is either excited directly, or when exciton transfer from other Chl (or Pheo) molecules to P680 is considerably faster than the decay of P680*. Exclusive excitation of P680 via *slow* exciton transfer from accessory Chl (or Pheo) molecules would lead to a very small, and even negative amplitude for the ultrafast charge separation component. This means that with the particles used in the previous study [4] the extent to which P680 was excited via slow energy transfer must have been rather low due to the loss of 2 of the accessory Chl molecules in the long-time Triton-treated particles [24,26], and/or due to a Triton X-100 induced disruption of (slow) energy transfer from accessory Chls to P680 [7]. The mere presence of about 2 additional Chls would lead to a drastic decrease in the relative amplitude of the previously resolved ultrashort component in the present preparation. This implies that excitation of P680 in the wavelength range from 620 to 635 is not very selective. Our interpretation is indeed supported by the fact that in the present RC preparation the about ~ 20 ps fluorescence component attributed to accessory Chl transferring its energy to, ultimately, P680 (see below) has an 8-times larger amplitude than the corresponding component in the previously studied longer Triton-exposed complexes (cf. Table I) [4]. This confirms that in the previously studied RC complex the fraction of the excitation reaching P680 via relatively slow energy transfer, as compared to the fraction of direct P680 excitation, was much lower. This difference causes the ultrashort fluorescence component, reflecting the direct charge separation process, to be reduced in amplitude drastically, and therefore it could not be resolved here.

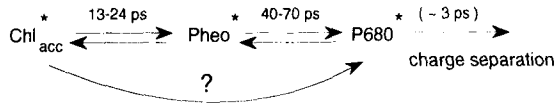
Energy transfer: involvement of three species in sequential transfer processes

The 15–32 K experiments (vide supra) indicate that an energy transfer process occurs from an energy donating species with an emission maximum around 675 nm, to an energy accepting species with an emission maximum around 681–684 nm. Based on its spectral properties [18], one or more accessory Chl *a* (i.e. those Chls a not constituting the primary donor P680) is the most likely candidate for the energy donor. The rate

constant of this process seems to be temperature-dependent, getting faster with lower temperatures: from a lifetime of 22 ps at 150 K (Fig. 3) down to 13 ps at 15 K (vide supra). The reason for this temperature dependence is unclear at present. The lifetime of 13 ps at 15 K for the presumed accessory Chl *a* is in agreement with recent optical hole-burning studies at 1.6 K, calculating a lifetime of 12 ps for the accessory Chl *a*, irrespective whether Triton X-100 or lauryl-maltoside/$LiClO_4$ has been used for the isolation [6,7].

In principle both Pheo *a* and P680 could be possible candidates for the energy accepting species, based on their presumed spectra [18]. However, we can exclude P680 as the main primary acceptor. If accessory Chl would transfer its energy solely to P680, a rise term in the fluorescence decay profile would be negligibly small also at very low temperatures, because the state P680* would decay much faster (by charge separation within a few ps) than it would be populated (energy transfer with a lifetime of 13–22 ps). The significant rise term can be explained only by assuming that to a substantial extent accessory Chl transfers its energy to a species different from P680 although an additional direct transfer route can not be excluded. As Pheo *a* is thought to have a slightly blue-shifted spectrum, as compared to P680 [5,6,18], we tentatively attribute the second lifetime component (40–70 ps; Fig. 6) to Pheo *a*, transferring its energy to P680. This is supported by the shape and maximum position of the 66 ps component (Figs. 3 and 5). The rise term due to the transfer from Pheo can not be detected in the fluorescence profile of P680* for the same reason as discussed above for the shorter-lived Chl component. Thus, the DAS of the 40–70 ps component shows only positive amplitudes as well. The energy transfer time from Pheo *a* to P680 of 40–70 ps is again in good agreement with hole-burning results, reporting a 50 ps lifetime for Pheo *a*, irrespective of whether Triton X-100 or lauryl-maltoside/$LiClO_4$ has been used for the isolation [6,7].

In conclusion, the fluorescence kinetic data suggest that accessory Chl *a* transfers its energy to Pheo *a* with a temperature-dependent lifetime of 13 ps (15 K)–22 ps (150 K). In turn, Pheo *a* probably transfers its energy to P680, with a lifetime of 40–70 ps. An alternative explanation for the 40–70 ps energy transfer step is the relatively slow relaxation of partly decoupled antenna Chls. The lifetime of this process is not very temperature-dependent, but it cannot be resolved well at all temperatures (see Fig. 4). All processes are summarized in Scheme I. This scheme is equivalent to the one already suggested by Small and co-workers on the basis of hole-burning studies [6,7]. It applies to all PS II RC preparations studied thus far (this report, and Refs. 4,6,7), even when they were analyzed in the presence of 0.05% Triton X-100 [15]. The rise term of



Scheme I. Schematic diagram of the energy transfer and charge separation processes in RC particles of PS II. The range of lifetimes given for energy transfer represent the equilibration times between these pigments in the temperature range from about 10 K up to room temperature. The charge separation time of about 3 ps put in parentheses has not been resolved directly in this work.

the 13–22 ps component could not be detected at higher temperatures in our measurements (see Figs. 3 and 4, and Ref. 4). A possible explanation for this behavior could be that the rate of backward energy transfer (i.e., from Pheo *a* to accessory Chl) becomes about as fast as the rate of forward energy transfer upon raising the temperature, thus resulting in a DAS that is the positive sum of the emission bands of both the energy donor and the acceptor.

Lifetimes of radical pair states

The DAS of the components with lifetimes varying from a few hundreds of picoseconds (Figs. 3 and 4) to about 50 ns (Fig. 7) all appeared very similar with a peak at about 682 nm, suggesting P680* as the origin. This also applies to the component with a lifetime of about 5–6 ns. In less gently treated PS II complexes, a blue-shifted 6 ns lifetime is usually observed, suggesting a contribution from uncoupled Chl emitting at a shorter wavelength than P680. The fact that in the present study the spectrum of this component is very similar to that of the other lifetimes (in the hundreds of ps and ns time-range) suggests that uncoupled Chl does not, to any significance, contribute to the fluorescence kinetics in our preparations studied here. It could very well be that some or all of the resolved components with lifetimes above 100 ps reflect radical pair recombination processes of radical pair states with different lifetimes. Whether these radical pair states are populated sequentially (several radical pair relaxation steps) or in parallel (population of different conformational substates of the RC and/or charge separation along two functionally different branches) remains to be clarified.

Recently Booth et al. [14] also reported on the presence of multiple radical pair states in stabilized long-term Triton-exposed RC complexes, with lifetimes ranging from 20 to 85 ns. It is interesting to note that for low temperature experiments, the samples used in that study were diluted with 50% glycerol [14], whereas in our experiments no cryoprotector was added. Apparently,. in both kinds of sample and under different conditions, multiple radical pair states are observed. This applied also to samples in the liquid state (277 K [14]), suggesting that freezing artifacts cannot be the reason for the multiexponential recombination kinetics.

Conclusion

In summary, we conclude that the charge separation in all the different RC complexes probably takes place within a few picoseconds, despite the fact that the corresponding fluorescence lifetime component could only be resolved in the stabilized and long-time Triton-exposed RC [4] thus far. Accessory Chl *a* seems to transfer its excitation energy mainly to Pheo *a* within 10–20 ps. The corresponding rise term in the fluorescence profile of Pheo *a* is only present at sufficiently low temperatures, where backward energy transfer is sufficiently slow. In turn, Pheo *a* probably transfers its energy to P680 within 35–75 ps. The resolution of the corresponding DAS is complicated by the only slightly different spectral properties of Pheo *a* and P680, as well as by the changes of the DAS corresponding to the energy transfer from accessory Chl *a* to Pheo *a*. We conclude that the observed short time (below 100 ps) excited state kinetics in the RC particles studied here is dominated by relatively slow energy transfer processes if the accessory Chls are excited preferentially. The effective rate of charge separation then becomes limited by the energy transfer steps when accessory Chl is initially excited. Thus the difference in the short-time kinetics as compared to the previously studied RC particles is easily related to the lower Chl content of the latter. This also rationalizes the observation of a slow (21 ps) Pheo reduction time, which was interpreted as the intrinsic charge separation time from P680 in a recent work [9]. We in turn assign this ≈ 20 ps component to an energy transfer limited charge separation.

The identification of the longer lifetime components, ranging from several hundreds of picoseconds to 50 ns, as charge recombination fluorescence, needs to be examined in more detail in the future. In addition to the unresolved controversy of discrete exponential lifetimes versus a continuous distribution of lifetimes [29], the analysis of the radical pair and charge recombination kinetics might be further complicated by spin-dephasing processes [30]. Spin-polarized triplet EPR spectra of P680 have been reported [31,32] and substantial triplet yields were estimated [11,33]. Singlet–triplet radical pair transitions, occurring on a nanosecond time scale, do not in fact exhibit simple first-order kinetics and might cause artifacts in analyses in which first-order kinetics are assumed.

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Recent experiments using D1-D2 RCs with 6 Chl per 2 Pheo and applying more selective excitation of P680 at 690 nm have revealed an approximately 3 ps component which is assigned to charge separation [34]. This fully supports our hypothesis made in this paper that the primary charge separation takes place within a few picoseconds in both types of RC complex.

A very recent paper by Durrant et al. [35] shows that the energy transfer between the chromophores of the RC takes place within a time of about 100 fs. Those authors could not resolve the longer-lived energy transfer components reported in this paper, however, due to their short measuring timescale of only 2 ps. In the light of their results, it may be possible that all energy transfer components reported in this work should be attributed to energy transfer from the two additional Chls present in the 6 Chl per 2 Pheo preparation used here. In particular, the 40–70 ps energy transfer component in our data would then also have to be interpreted as energy transfer starting on one or both of these additional Chls, as pointed out above as one alternative possibility as compared to the involvement of a Pheo-excited state in this process. The energy transfer processes with relatively small amplitudes found by us in RCs with about 4 Chl per 2 Pheo [4] probably have to be attributed to a small amount of RCs (less than 10%) carrying more than 4 Chl per 2 Pheo in that preparation. This is in line with the hypothesis put forward in this paper.

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