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Energy transfer and trapping of excitations in membranes of *Heliobacterium chlorum* at 15 K

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The transfer of excitation energy in membrane fragments of *Heliobacterium chlorum* was studied at 15 K by picosecond transient absorption spectroscopy. Due to the high repetition rate of the laser pulses, the primary electron donor was in the oxidized state in essentially all reaction centers. The absorbance difference spectrum showed that excitations in the antenna rapidly accumulate on the long wavelength absorbing component bacteriochlorophyll (BChl) *g* 808. Analysis of the kinetics of excited antenna BChl *g* showed a lifetime of less than 1 ps for excitations on BChl *g* 778, whereas excited BChl *g* 793 showed a somewhat slower decay of about 2 ps. Time resolved anisotropy measurements showed a very rapid randomization of excitations amongst pigments with similar absorption spectrum within less than 1 ps. Kinetics as well as the absorbance difference spectrum showed that the decay of excitations accumulated on BChl *g* 808 was strongly inhomogeneous. About 50% of the excitations disappeared with a time constant of 4 ± 2 ps, while the others showed decays with time constants of 20 ± 5 ps and 100 ± 20 ps. The amplitude of the 20 ps component decreased with the energy density of the excitation pulses and this decay component is attributed, at least in part, to singlet-triplet quenching. The main relaxation process of excitations with a time constant of about 4 ps is most probably due to energy transfer to the oxidized reaction center. The 100 ps decay component is associated with BChls *g* 808 that are relatively isolated, either spatially or energetically.

Introduction

Heliobacterium chlorum belongs to a separate division of photosynthetic bacteria, the heliobacteria, and contains, both in the antenna and in the reaction center, a new type of bacteriochlorophyll, BChl *g* [1,2]. The antenna and reaction center pigments are bound to a single pigment-protein complex, with about 35 antenna BChls *g* per reaction center [3,4]. The absorption spectrum at 6 K reveals three different spectral components, absorbing at 778, 793 and 808 nm, respectively [5]. The primary electron donor, P-798, is thought to be a dimer of BChl *g* [6] or of its 13^2-epimer [7] and shows a maximum bleaching at 798 nm upon oxidation at room temperature [8,9]. The primary electron acceptor has been identified as 8^1-hydroxychlorophyll *a* [4] and shows an absorption maximum near 670 nm.

Like many other photosynthetic organisms, heliobacteria contain an antenna component absorbing at longer wavelength than the reaction center. This long wavelength component is thought to be located near the reaction center in order to achieve a high concentration of excitations to increase the probability of trapping (see, for example, Ref. 10). At room temperature excitations equilibrate rapidly among the three antenna components and are trapped by P-798 with a time constant of 20–25 ps [11–13]. The rate of disappearance of the excitations was found to be equal to the rate of formation of the reduced primary acceptor, indicating that charge separation by itself is a very rapid process [11].

At 6 K, fluorescence measurements and time resolved absorption difference measurements showed that excitations in the antenna of *H. chlorum* accumulate on the long wavelength absorbing antenna component, BChl *g* 808 [5,14]. These excitations are transferred to the reaction center with about 70% efficiency [15]. It has been shown by Deinum et al. [16] that, at low temperature, quenching of BChl *g* 808 excitations by the oxidized reaction center (P-798$^+$) is almost twice as efficient as by the reduced reaction center (P-798). Time resolved fluorescence measurements at 25 K showed that BChl *g* 808 is strongly inhomogeneous, but did not provide definite information on the

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Abbreviations: BChl, bacteriochlorophyll; fwhm, full width at half maximum; P-798, primary electron donor.

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rate of trapping of excitations by the reaction center, either in the reduced or oxidized state [17].

In this paper it will be shown that at 15 K, excitations accumulate on the long wavelength absorbing antenna component BChl *g* 808 in 2 ps or less. In particular, energy transfer from BChl *g* 778 to pigments absorbing at longer wavelengths was very fast and could not be resolved within the experimental time-resolution. At least three decay components were observed in the decay of excited BChl *g* 808. The most rapid of these (4 ps) is ascribed to energy transfer to the oxidized reaction center.

Materials and Methods

Heliobacterium chlorum was grown anaerobically in medium No. 1552 of the American Culture Collection and membrane fragments were prepared as described by Van de Meent et al. [3]. The membrane fragments were suspended in a buffer containing 10 mM Tris and 30 mM ascorbate (pH 8.0) with 60% glycerol (v/v) to maintain a clear sample at low temperature. The absorbance was about 0.8/mm at 788 nm at room temperature. Samples were cooled in the dark in an Oxford CF 1204 helium flow cryostat in a cuvette with an optical pathlength of 1.2 mm. The temperature in the sample was measured by a calibrated Siemens BAV 99 diode [18] and adjusted to 15 K, unless otherwise indicated.

Time resolved transient absorption experiments were performed in so-called 'one-color' or 'two-color' pump-probe experiments using one or two synchronously pumped dye lasers, respectively, operating at 76.6 MHz [11]. The dye lasers covered the spectral range from 750 to 840 nm with two different dyes, LDS 751 and LDS 821, producing linearly polarized picosecond pulses. In one-color pump-probe experiments the output of the dye laser was split into an excitation and a probe pulse, in which case the instrumental time-response was determined by the autocorrelation trace with typical fwhm of 4–6 ps. Two-color pump-probe experiments were performed using two dye lasers resulting in an instrumental time-response of about 15 ps, equal to the cross-correlation of the two dye laser pulses.

The probe beam was passed through a variable delay line and was focused into the sample in overlap with the excitation beam. The measurements were performed with a typical laser energy of 5 mW, corresponding to an energy density in the sample of about 5 μJ/cm^2 per pulse; the energy of the probe beam was less by a factor of 20. The transient absorption signal was recorded using a double modulation technique and homodyne detection with a detection limit for absorbance changes in the order of 10^{-5} [11]. The excitation beam was modulated at 8 MHz using an acousto-optic modulator, while the probe beam was modulated at 2.5 kHz using a mechanical chopper. Kinetic data were obtained by recording the transient absorption signal as a function of the delay between excitation and probe beam. Transient absorption spectra were obtained by tuning the probe laser and recording the signal at a fixed delay after excitation.

Absorption kinetics as a function of polarization of the probe beam with respect to the excitation beam were recorded by changing the relative polarization from parallel to perpendicular, using a birefringent filter combined with a Glan-Thompson polarizer. The observed anisotropy $r(t)$ is defined as:

$$r(t)=(\Delta A_{\parallel}(t)-\Delta A_{\perp}(t))/(\Delta A_{\parallel}(t)+2\Delta A_{\perp}(t))$$

Kinetic transients of the accumulation and decay of charge separated and triplet states were recorded by square-wave modulation of the intensity of the excitation beam at 60–250 Hz, using the acousto-optic modulator. The sample was illuminated during several milliseconds with a sequence of excitation pulses exciting the sample at the repetition rate of 76.6 MHz. This resulted in accumulation of charge separated and triplet states, which have lifetimes in the ms and μs time region. Time constants of the accumulation and decay of these long-lived states could be measured by monitoring the intensity of the probe beam with a time-resolution of 20 μs, determined by the time-response of the detector. The intensity of the probe beam was recorded with a photodiode (HUV 4000 B) and digitized using a Le Croy 9410 oscilloscope.

Results

Energy transfer in the Q_y absorption band

Time resolved absorption difference spectra of membranes of *H. chlorum* at 15 K upon excitation at 797 nm are shown in Fig. 1A. The repetition frequency of excitation flashes (76.6 MHz) was such as to ensure almost complete decay of excited singlet states between excitation flashes. The excitation energy density was about 10^{13} photons per cm^2 per flash, well below the energy density where singlet-singlet annihilation may occur [19]. The time between excitation flashes was too short, however, for a significant decay of long-lived components like triplet states in the antenna and charge separated states in the reaction center [20]. In fact, with the excitation energy densities used in our experiments, essentially all reaction centers were in the oxidized state (see below).

The first spectrum of Fig. 1A (triangles) shows the absorption difference spectrum at a delay of 10 ps after excitation and shows a maximum bleaching at 812 nm. The second spectrum (solid circles), obtained at a delay of 30 ps after excitation shows a maximum bleaching at

814 nm, while the third spectrum (open circles) was obtained at a delay of 110 ps after excitation and shows a maximum bleaching at 816 nm. The excitation wavelength (797 nm) was chosen such that mainly the BChl *g* 793 antenna component (see Fig. 1B) was excited. Nevertheless, comparison of the absorption difference spectra with the absorption spectrum (Fig. 1B) shows that the bleaching in the absorption difference spectrum reflects almost exclusively the formation of excitations on the long-wavelength absorbing antenna component BChl *g* 808. This indicates that within a few ps all excitations have accumulated on BChl *g* 808. This is consistent with earlier measurements at lower time resolution [14] and is also in agreement with the observation that essentially all fluorescence originates from BChl *g* 808 [5,17]. It can be seen that the maximum bleaching of the absorption difference spectrum shifts to longer wavelength at longer delay time after excitation. This means that the lifetime of the excited state varies along the Q_y absorption band of BChl *g* 808.

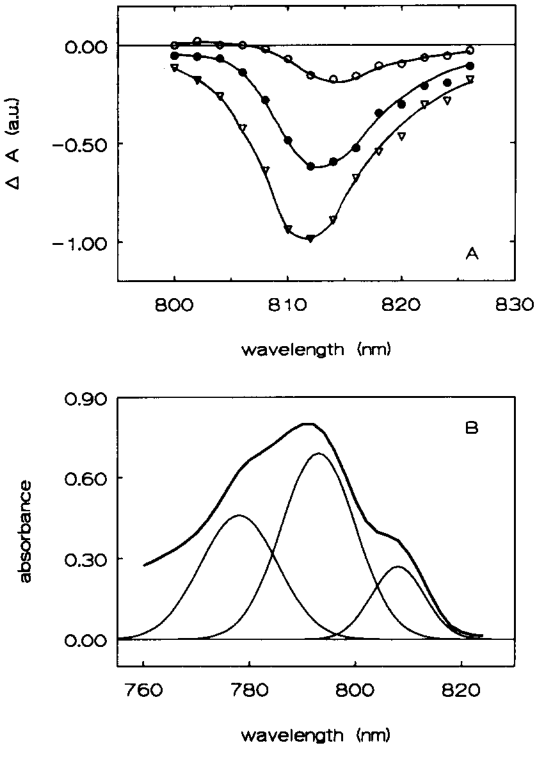

Fig. 1. Absorption difference spectra (A) and absorption spectrum (B) of membranes of *Heliobacterium chlorum* at 15 K. The absorption difference spectra were recorded at a delay of 10 ps (▽), 30 ps (●) and 110 ps (○) upon excitation at 797 nm. The excitation energy density was about 6 $\mu J/cm^2$ (10^{13} photons per cm^2) per flash and essentially all reaction centers were in the oxidized state. The absorption spectrum shows the bands of BChl *g* 778, BChl *g* 793, and BChl *g* 808, obtained by gaussian deconvolution according to van Dorssen et al. [5]. a.u.: arbitrary units.

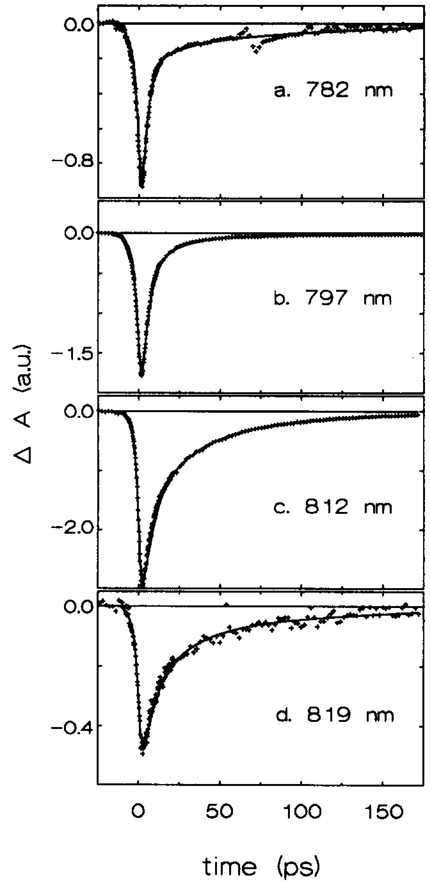

Fig. 2. Kinetics of the bleaching in the Q_y band of BChl *g* at 15 K at 782 (a), 797 (b), 812 (c) and 819 nm (d) in one-color pump-probe experiments. The results of an exponential fit (solid line) are summarized in Table I. The excitation energy density was between 3 and 11 $\mu J/cm^2$ per flash. The curves were divided by the number of absorbed photons to correct for differences in absorption and excitation energy density.

These features are also observed in the kinetics of the absorption changes in one-color pump-probe experiments, shown in Fig. 2. In agreement with fluorescence lifetime experiments [17], we observed a strongly inhomogeneous decay of excited antenna pigments. Upon excitation at 782 nm mainly antenna pigments of the short-wavelength absorbing antenna component, BChl *g* 778, are excited. We observed a small initial bleaching which rapidly decayed to the ground state. The kinetic trace at 782 nm was fitted to an exponential function with time constants of 1.5 ps (91%), 15 ps (5%) and 100 ps (4%) (see also Table I). When plotted for the same number of absorbed photons, the amplitude of the initial bleaching was about two times smaller than at 797 nm (recording 2b). At wavelengths shorter than 782 nm the amplitude of the initial bleaching rapidly decreased (data not shown) and almost no signal was observed below 775 nm, in spite of the fact that there is still considerable absorption at this wavelength. Although the effect may in part be due to excited state absorption, this indicates that both the amplitude and the decay time of the bleaching are

TABLE I

Lifetimes τ_i (1/e) and amplitudes A_i of decay components at selected wavelengths, obtained in 'one-color' pump probe experiments at 15 K

The energy densities are those of the excitation pulses. The time constants are given in ps with an uncertainty of ±20%, their total amplitude was normalized to 1.

Wavelength (nm)	Energy density ($\mu J/cm^2$/flash)	τ_1	A_1	τ_2	A_2	τ_3	A_3
782	3	1.5	0.91	15	0.05	100	0.04
797	10	2.1	0.88	15	0.11	100	0.01
812	11	4.0	0.63	23	0.26	80	0.10
819	9	4.0	0.56	20	0.31	100	0.13

limited by the time resolution of our apparatus, and that the true time constant of the rapid decay phase may be up to an order of magnitude smaller than indicated by our analysis.

At 797 nm a rapid decay component of 2 ps was observed, which in this case could be well resolved because of a high signal-to-noise ratio, together with the same exponential components as at 782 nm, but with somewhat different relative amplitudes. At 812 nm and 819 nm (recordings 2c and 2d) the 2 ps component was replaced by one of 4 ps and the relative amplitudes of the slower components were considerably larger (Table I).

Fig. 3 shows the decay associated absorption spectrum obtained from a fit with 4, 20 and 100 ps components as determined by one-color experiments like those in Fig. 2. The 4 ps component showed a maximum at 808 nm, and resembles the BChl *g* absorption spectrum (Fig. 1B). The other components were more red-shifted, in general agreement with the results shown in Fig. 1A.

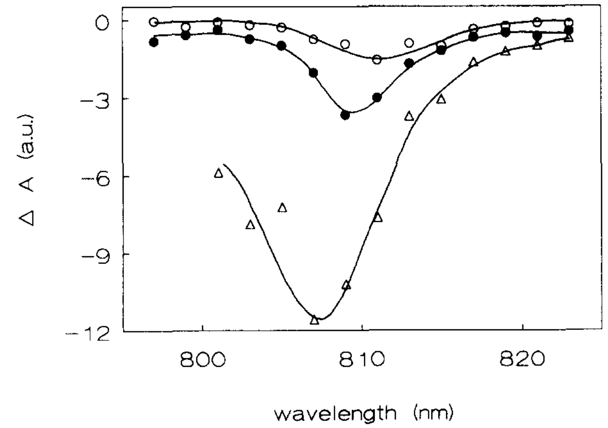

Fig. 3. Decay associated absorption difference spectra obtained by fitting the kinetics with exponential components of 4 ps (△), 20 ps (●) and 100 ps (○). The kinetic data were obtained in one-color pump-probe experiments as shown in Fig. 2. The spectra were divided by the number of absorbed photons per flash to correct for the different excitation energy densities (3–6 $\mu J/cm^2$ per flash) and absorption at each wavelength.

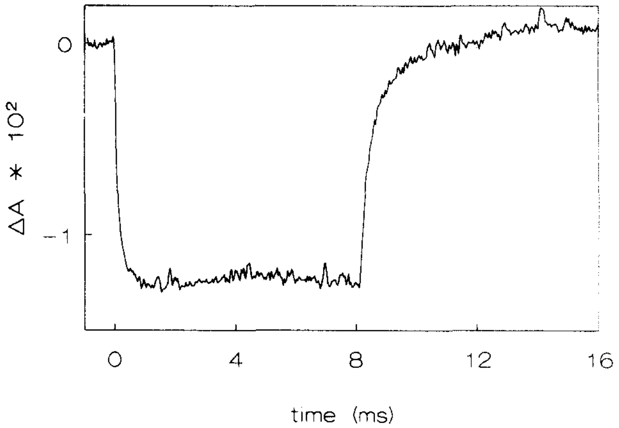

Fig. 4. Kinetics of absorbance changes at 810 nm due to accumulation of long lived states upon excitation of the sample during a 8 ms period. The decay kinetics were fitted to a bi-exponential function with time constants of 350 ± 50 μs (72%) and 2.3 ± 0.2 ms (28%). The excitation energy densities of the excitation and the probe flashes were about 0.4 $\mu J/cm^2$ and 0.003 $\mu J/cm^2$ per flash, respectively.

Accumulation of antenna triplets and the origin of the 20 ps decay component

Under the conditions of our experiments not only the accumulation of P-798$^+$ but also of antenna triplets may be expected [20]. In order to obtain information on the extent of triplet accumulation, the bleaching at 810 nm was measured in experiments where the excitation beam was modulated at 60 Hz (see Materials and Methods). Fig. 4 shows the time dependence of the absorbance of the sample during one period of the modulation frequency. A bleaching occurred upon illumination of the sample by the excitation beam, followed by a reversal upon cessation of illumination. The difference spectrum (Fig. 5) resembles the antenna triplet spectrum that was reported previously and may

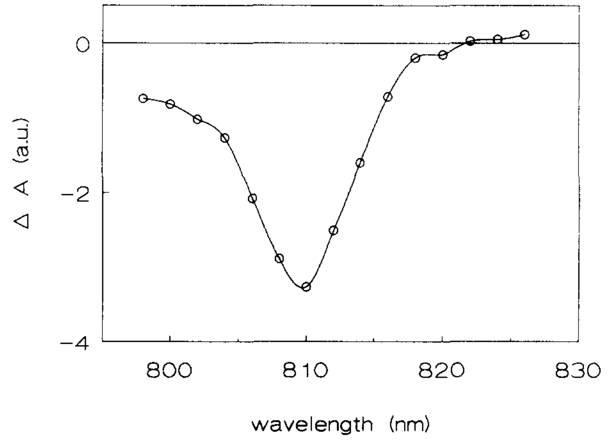

Fig. 5. Difference spectrum of the absorbance changes due to triplet formation. The modulation frequency of the excitation beam was 250 Hz. The excitation energy densities of the excitation and the probe flashes were 0.4 $\mu J/cm^2$ and 0.03 $\mu J/cm^2$ per flash, respectively. The spectrum was divided by the number of absorbed photons per flash to correct for differences in absorption and excitation energy density.

be attributed to the generation of triplet states of BChl *g* 808 [20,21], which accumulate already in significant amounts at relatively low energy densities.

At relatively high energy densities of the probe beam (Fig. 5) neither the difference spectrum, nor the decay kinetics showed a contribution by P-798$^+$ and a rough calculation indicated that the intensity of the probe beam was sufficient to keep essentially all P-798 in the oxidized state. However, at lower probe beam intensity (Fig. 4) a biphasic decay was observed with a component of 350 μs (70% of the total amplitude), due to the antenna triplets and one of 2.3 ms, which can be attributed to P-798$^+$ re-reduction [20].

In order to estimate the effect of singlet-triplet quenching on the decay of the BChl *g* singlet bleaching, we performed time-resolved absorption difference measurements at 812 nm at two different energy densities. At an energy density of 4 μJ/cm^2 per pulse, comparable to those of Fig. 2, we observed the normal decay profile, which could be analyzed in the three components of 4, 20 and 100 ps. At 15–20 times lower energy density the amplitude of the 20 ps component was approximately halved, while the amplitude of the 100 ps component was almost doubled. In both experiments essentially all reaction centers were in the oxidized state. This indicates that the 20 ps component is, at least partly, due to singlet-triplet annihilation.

Time resolved anisotropy

All results presented so far were obtained with pump and probe pulses that had a parallel polarization with respect to each other. This means that the decay components in experiments such as those of Fig. 2 might in part be due to a decrease of the anisotropy of the sample after the flash, due to energy transfer from the initially excited BChls to pigments molecules with similar absorption spectrum but with different orientation. In order to check this possibility we compared the kinetics of the bleaching with probe flashes that had either a parallel or a perpendicular polarization with respect to the pump flash.

Fig. 6 shows the results of such an experiment at 799 nm. For both directions of polarization the kinetics were essentially the same, but with perpendicularly polarized flashes the amplitude of the signal was approximately a factor of two smaller, corresponding to an anisotropy r with a constant value of 0.2 throughout the experiment. Since the anisotropy, r, is related to the angle θ between the transition moments of two chromophores by the relation $r = (3\cos^2\theta - 1)/2$, our data indicate that the average angle between antenna pigments absorbing at the same wavelength is about 35°.

Our results, which resemble those of e.g., Gillbro et al. [22] with chlorosomes, indicate a very fast randomization of the excited states among molecules with similar absorption spectrum. This is then followed by a relaxation of the excitations by energy transfer to antenna pigments absorbing at longer wavelength, or by other quenching processes, including singlet-triplet quenching, as mentioned above.

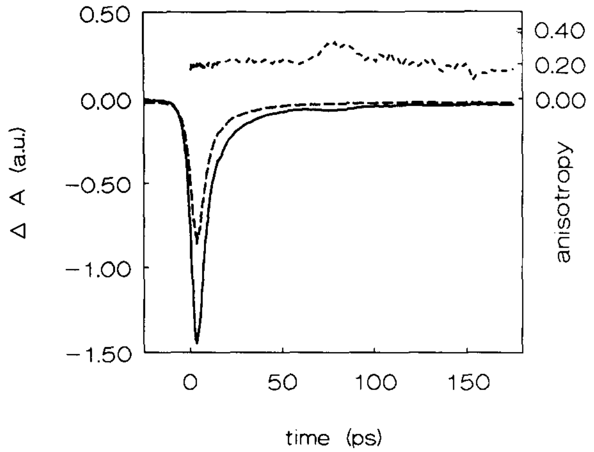

Fig. 6. Kinetics of the absorption difference signal at 799 nm at 4 K. The probe beam was polarized parallel (solid line) or perpendicular (dashed line) to the polarization of the excitation beam. The excitation energy density was about 10 μJ/cm^2 per pulse. The broken line (top) represents the calculated anisotropy $r(t)$.

Discussion

The results reported in this paper demonstrate the existence of very rapid energy transfer and quenching processes in the antenna-reaction center complex of heliobacteria at low temperature. Very fast relaxation processes occur upon excitation in one of the short wavelength absorbing BChls *g*, leading to a randomization of the excitations amongst neighboring BChls followed by a rapid decay. In particular for BChl *g* 778 this decay appears to be very fast and may occur with a time constant of only a few hundred femtoseconds. The rate of decay is somewhat slower for BChl *g* 793, but may still be larger than indicated by the time constant of 2 ps given in Table I, which may be instrument limited. Since the fluorescence excitation spectrum shows highly efficient energy transfer from BChl *g* 778 and BChl *g* 793 to BChl *g* 808 [3,5], we ascribe these decay processes to energy transfer to BChl *g* 808. Similarly high rates have been observed in the core antenna of Photosystem I of plants [23]. It may be noted here that the rates of transfer are too high to be observed as a growing in of the bleaching in the two-color pump-probe experiments of Fig. 1A.

Our results, like those of Kleinherenbrink et al. [17], indicate that the BChl *g* 808 antenna is quite inhomogeneous. A dominant, rapid decay component of 4 ps, was observed in BChl *g* 808, together with slower decay components of about 20 ps and 100 ps. These components appear to correspond to the 27 and about 100 ps decay components observed at 25 K in the decay associated fluorescence emission spectrum [17]. Although part of the signal at longer wavelengths may be due to stimulated emission, the absorption spectra of these components are clearly red shifted with respect to that of the most rapid component and the same applies to the fluorescence maximum of the 100 with respect to the 27 ps component [17]. The 4 ps component was not observed in the fluorescence decay [17], which may be explained by a limitation imposed by the time response of the instrument. It may be noted that long-lived components of approx. 300 and 900 ps with even more red shifted emission spectra were also observed in the fluorescence decay [17]. Apart from the fact that single-photon counting may provide a more sensitive method to observe such long-lived components than do absorption measurements, it may be remarked that the one-color experiments were not well suited to detect such long-wavelength components, since the signal to noise ratio decreased progressively beyond 812 nm (see Fig. 2) due to a decrease in the photon absorption at longer wavelengths.

Since the excitation spectrum shows that, even at low temperature, most excitations in BChl *g* 808 are effective in bringing about charge separation [17] and since singlet-singlet annihilation can be ruled out in our experiments, we tentatively assume that the 4 ps decay component is, at least mainly, due to energy transfer to the reaction center, which is in the oxidized state (P-798$^+$) in our experiments. It should be noted here, that Deinum et al. [16] observed that, at low temperature, P-798$^+$ quenches BChl *g* fluorescence almost twice as effective as does the photoactive form, P-798. The possible mechanism of this quenching has been discussed in terms of the Förster mechanism [16]. The rate of transfer to open reaction centers would probably be lower, but still considerably faster than the overall rate of energy transfer to the reaction center at room temperature [11]. As mentioned above, the 20 ps decay appears to be associated, at least in part, with singlet-triplet quenching.

With a total of about 35 BChls *g* per reaction center [3,24] the relative amplitude and width of the 808 nm absorption band (Fig. 1B) suggests that there are only about 4–5 BChl *g* 808 molecules per reaction center, while experiments of Deinum et al. [19] indicate that there is very little energy transfer between different photosynthetic units at low temperature. The picture that emerges from the lifetime measurements thus results in a model where 2–3 BChls *g* 808, as judged from the amplitude of the 4 ps component, are closely coupled to the reaction center, while the other BChl *g* 808 molecules, absorbing at somewhat longer wavelengths, are in a more isolated position, spatially or energetically, both with respect to the reaction center and with respect to each other. The latter is also in agreement with the fluorescence polarization spectrum, which shows a strong increase on the long-wavelength side of the BChl *g* 808 band [5] at low temperature.

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