Biochimica et Biophysica Acta 850 (1986) 501–512
Elsevier

BBA 42084

Singlet–singlet annihilation at low temperatures in the antenna of purple bacteria

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(Received February 4th, 1986)

Key words: Energy transfer; Singlet–singlet annihilation; Antenna structure; Bacterial photosynthesis; (*R. rubrum, Rps. sphaeroides*)

Chromatophores of the purple photosynthetic bacteria *Rhodospirillum rubrum* and *Rhodobacter (Rhodopseudomonas) sphaeroides* were excited by means of 35-ps flashes at 532 nm of varying intensities, both at room temperature and at 4 K. With increasing exciting energy densities the integrated yield of fluorescence produced by these flashes was found to decrease considerably due to singlet–singlet annihilation. An analysis of the results showed that in *R. rubrum* the number of connected antenna molecules between which energy transfer is possible decreases from about 1000 to about 150 when the temperature is lowered from 298 to 4 K. In *Rb. sphaeroides* the B875 light-harvesting complex appears to contain about 100 connected bacteriochlorophyll (BChl) 875 molecules at 4 K, while the B800–850 complex contains about 45 BChl 850 molecules. The data are explained by a model for the antenna of *Rb. sphaeroides* in which units of B875, containing about four reaction centres, are separated by an array of B800–850 units that surrounds B875. By applying a random walk model we found that in both species the rate of energy transfer between neighbouring antenna molecules decreased about 10-fold upon lowering the temperature. The rate of energy transfer from antenna molecules to either open or closed reaction centres decreased only 3- to 4-fold in *R. rubrum* and remained approximately constant in *Rb. sphaeroides* upon cooling. A blue shift of the emission spectra at 4 K of both species was observed when the excitation energy density was increased to a level where singlet–singlet annihilation plays a significant role. This observation appears to support the notion that an additional long-wave pigment exists in the antenna of these bacteria.

Introduction

The transfer of excitation energy in photosynthesis is a well-established and extensively studied phenomenon [1]. Two fundamental concepts have emerged. The first is that for each reaction centre a large number of antenna pigments transfer their excitation energy efficiently to these reaction centres [2]. This assembly of a reaction centre with its associated antenna pigments is called a photosynthetic unit. The second is that in many photosynthetic systems a large number of photosynthetic units are interconnected, i.e., the ensemble of antenna pigments may be viewed as a large 'lake' that is shared by several reaction

Abbreviations: BChl, bacteriochlorophyll; BChl 800, BChl 850 and BChl 875, bacteriochlorophyll absorbing around 800, 850 and 875 nm, respectively; B800–850 and B875, antenna pigment-protein complexes containing BChl 800 and 850 and BChl 875, respectively; I, primary electron acceptor (bacteriopheophytin *a*); P, primary electron donor; Q, first quinone acceptor.

centres. The reaction centres thereby act as traps for the excitations. In that case one of the major effects that can be observed is that if a reaction centre that is inactive or 'closed' is visited by an excitation, this excitation may escape and be trapped by a neighbouring, still open, reaction centre [3]. This phenomenon results in the well-known linear experimental relationship between the reciprocal of the fluorescence yield and the fraction of closed traps that was observed in many systems [4,5], but only recently theoretical derivations have been given [6,7].

The concept of domains has been introduced by Clayton [8] as the cluster of connected antenna pigments among which excitations can be transferred freely; transfer between pigments belonging to different domains is severely restricted. One way to determine the sizes of these domains is to measure the quenching of the integrated fluorescence yield as a function of the intensity of a picosecond laser flash [9]. When more than one excitation is present in a domain, the annihilation of two singlet excitations causes the number of excitations to decrease, and therefore the fluorescence yield to drop.

A general descripton of the relation between the integrated fluorescence yield $\phi_f^c(z)$ of a system with oxidized reaction centres and the average number of excitations created in a single domain upon delta pulse excitation (z), was first derived by Paillotin et al. [9]:

$$\phi_f^c(z) = \phi_f^c(0) r \sum_{k=0}^{\infty} \frac{(-z)^k}{r(r+1)(r+2)\ldots(r+k)} \frac{1}{k+1} \qquad (1)$$

In this expression, r is a parameter that expresses the efficiency of annihilation relative to mono-excitation decay processes:

$$r = \frac{2\gamma_1}{\gamma_2} \qquad (2)$$

in which γ_1 is the rate of mono-excitation decay due to trapping or losses, averaged over a domain, and γ_2 is the rate of annihilation per pair of excitations in a domain. A high value of r thus corresponds to a low efficiency of annihilation.

In experimental annihilation curves the integrated fluorescence yield ϕ_f is plotted against the logarithm of the energy of the exciting laser flash, which energy is proportional to the variable z in Eqn. 1. The value of r determines the slope of such a quenching curve, as well as the fluorescence decrease caused by one excitation per domain. Since the energy, corresponding to one excitation per domain is needed for further calculations, r has to be determined from the slope of the quenching curve. In practice only r-values of not more than 2 can be distinguished by this method. When the value of r of an experimental curve has been determined, the energy corresponding to $z = 1$ is also known. Calibration of the intensity axis to the number of excitations generated in the system makes it then possible to calculate the number of antenna molecules, N_D, in a domain [9].

Exact expressions are available that relate γ_1, γ_2 and N_D to the rate constant for loss on an antenna molecule k_1, to the molecular rate constants for energy transfer between two neighbouring antenna molecules, k_h, for annihilation upon collision of two excitations, k_a, and for trapping k_t^o and k_t^c by open or closed reaction centres, respectively [6]. When trapping is allowed to occur simultaneously with annihilation, and thus the transition of reaction centres from the open to the weakly trapping or closed state P^+ has to be taken into account, the theoretical description of the process becomes more complicated. However, expressions for $\phi_f(z)$, the integrated fluorescence yield, and $q(z)$, the fraction of traps closed as functions of z, are available [6].

There is various evidence that domain sizes in purple bacteria, at least at room temperature, are large. Measurement of the fluorescence yield as a function of the fraction of traps in the state P^+ [4], of the magnetic-field-stimulated fluorescence yield as a function of reaction centres in the state Q^- (primary quinone electron acceptor Q reduced) [5], of the efficiency of quenching of singlet excitations by triplet states [10] support the lake model for energy transfer. This applies also to measurements of the yield of BChl fluorescence as a function of the intensity of the exciting picosecond laser flash [11–13], and its was shown recently that the effect of annihilation is also observed in the amplitude and kinetics of flash-induced absorbance changes due to formation and decay of excited BChl *a* in chromatophores of *Rhodospiril-*

lum rubrum [14]. Analysis of annihilation experiments by means of a model that will be discussed below gave a domain size of 10–20 connected photosynthetic units for *Rhodospirillum rubrum* and of more than 20 connected photosynthetic units for *Rhodobacter (Rhodopseudomonas) sphaeroides* [13]. Estimates for k_h and k_t yielded $k_h = (1.5 \pm 0.5) \cdot 10^{12}\ s^{-1}$ and $k_t^o = (5 \pm 1) \cdot 10^{11}\ s^{-1}$ for *R. rubrum* and $k_h = 4 \cdot 10^{11}\ s^{-1}$ and $k_t^o = 3 \cdot 10^{11}\ s^{-1}$ for *Rb. sphaeroides*. Measurements of fluorescence yields at low temperatures by Rijgersberg et al. [15] indicated that these rate constants decrease considerably upon lowering the temperature.

The experiments described here extend the annihilation measurements to low temperatures (4 K). Our results show that both in *R. rubrum* and *Rb. sphaeroides* a strong decrease of the domain sizes in the long-wave antenna complex, B875, occurs upon lowering the temperature. An analysis of the annihilation curves indicates that a model with a relatively slow energy transfer may explain the data. Measurement of the BChl 850 fluorescence vs. pulse intensity curve in *Rb. sphaeroides* at 4 K shows that the B800–850 complex consists of relatively small aggregates.

Materials and Methods

Rhodospirillum rubrum strain S1 was cultured in a chemostat as described elsewhere [12]. *Rhodobacter (Rhodopseudomonas) sphaeroides* strain 2.4.1 was cultured as described in Ref. 15. Chromatophores were prepared by sonifying the cells for 10 min at 0°C, followed by high speed centrifugation. For all experiments the chromatophores were suspended in a buffer containing 10 mM Tris (pH 8.0)/5 mM $MgCl_2$/0.5 M sucrose/55% (v/v) glycerol. The absorbance at the excitation wavelength never exceeded 0.1 to ensure a homogeneous light distribution within the sample.

Annihilation experiments were performed using a spectrofluorimeter described elsewhere [13]. The sample was excited by a frequency doubled Nd:YAG laser with a maximum pulse energy of about 5 $mJ \cdot cm^{-2}$ at 532 nm and a pulse width of approx. 35 ps. The sample could also be illuminated with continuous background illumination at 520 nm or with a xenon flash provided with narrow band filters passing either 420 or 520 nm. The time-integrated fluorescence and the intensity of the exciting flash were measured by means of photodiodes (RCA C30810). The diode that monitored the excitation intensity was calibrated by means of a glass absorber type laser energy meter (model 172R by Laser Instrumentation Ltd., U.K.).

The raw experimental data were compared to plots of Eqn. 1, generated by a computer, to estimate a value for the parameter r. For clarity of representation, however, the figures show the average of 10 successive data points. Before averaging, data points with a deviation towards higher yield which are caused by erroneous (double) pulses from the laser were removed using a computer regression method: from sets of 25–50 adjacent data points a best linear regression line was calculated, and data points with a deviation more than 20 times the standard deviation were removed.

Emission spectra were recorded using either a 35 ps laser flash at an incident energy density between 4 and 5 $mJ \cdot cm^{-2}$, or with a xenon flash at 0.1 $mJ \cdot cm^{-2}$. The resolution of the recording monochromator was set to 2 nm.

Results and Interpretation

Rhodospirillum rubrum

Fig. 1 shows the time integrated fluorescence yield induced by a 35 ps, 532 nm laser flash in chromatophores of *R. rubrum* at room temperature and at 4 K. In both cases the reaction centres had been brought in the state P^+ by illuminating the sample continuously during freezing and throughout the experiment. As mentioned already, the shape of the curves is not only determined by the optical cross section of the molecules at the wavelength of excitation and by the domain size, but also by r. For example, for $r = 0$ the intensity at which on the average one excitation per domain is generated already gives a fluorescence decrease to $1 - e^{-1}$ (i.e., about 37% quenching), whereas at higher r values (less efficient annihilation) the yield drops much less at the same energy (e.g., less than 15% quenching at $r = 2$). Therefore both curves were fitted to Eqn. 1 to obtain the best value for r. In this way r was found to be at least 2 at room temperature, which is larger than the

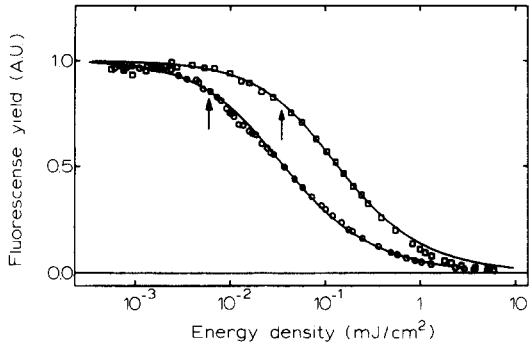

Fig. 1. Time-integrated normalized fluorescence yield of *R. rubrum* chromatophores as a function of the energy density of the exciting laser flash. For clarity of presentation, the averages of 10 successive data points are plotted. The reaction centres were kept in the oxidized state (P^+) by applying continuous background illumination. (○): Room temperature, fluorescence detected at 900 nm. (□): 4 K, 917 nm. The curves are normalized at low energies. The continuous curves are fits by means of Eqn. (1) with $r = 2$ for room temperature and $r = 1$ at 4 K (see text). A.U., arbitrary units.

value of 1.1, reported by Bakker et al. [13]. At 4 K we found $r = 1.0 \pm 0.3$.

The intensities at which on the average one excitation per domain is generated are indicated by arrows. It can be seen from Fig. 1 that a marked shift to higher pulse intensities occurs for the curve at 4 K, although the r-value decreases. These results can be used to calculate the size of the domains, using the absorbance of the sample, a room-temperature BChl extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm [16] and an energy-transfer efficiency from carotenoid to BChl 875 of 30%, both at room temperature and at 4 K [5]. For the low-temperature measurements the increase in sample concentration by shrinkage was estimated to be 5%. Our calculations yield a domain size $N_D \geqq 1000$ BChl 875 molecules at room temperature (assuming $r = 2$), and $N_D = 150$ BChl molecules at 4 K ($r = 1$). The value of N_D at room temperature is in agreement with the number obtained by Bakker et al. [13]. Since for r values higher than 2 the slopes of the curves become almost indistinguishable, larger values of r at room temperature (and consequently larger domain sizes) cannot be excluded and therefore the value of 1000 molecules must be regarded to be a lower limit. In a separate experiment the number of antenna molecules per reaction centre, N, was determined from the bleaching of P-870 in saturating continuous light and found to be 35 for this particular sample. This implies that at room temperature more than 25 photosynthetic units are connected, while at 4 K the domains have become partially disconnected and contain on the average only four reaction centres. $N_D \geqq 1000$ at room temperature and $N_D = 150$ at 4 K were also obtained for a sample that had been grown at low light intensity and which contained 65 BChl 875 molecules per reaction centre.

Fig. 2 compares annihilation curves at 4 K of chromatophores of *R. rubrum* in either the state $P^+I\ Q$ (●) (as in Fig. 1) or P I Q (○). At higher energies the fluorescence yield in the state P I Q drops less rapidly than in the state $P^+I\ Q$. This difference is caused by the fact that in the state P I Q at low energies, there are open reaction centres present keeping the yield low, while at higher energies these become progressively closed, giving rise to a relative increase in the fluorescence yield, partly counteracting the annihilation quenching [13]. We also measured the fluorescence intensity due to a weak probing xenon flash at 420 nm, given 1.5 ms after the laser flash. After this time the fluorescence due to the laser flash has decayed but at least 90% of P^+ created by the laser flash

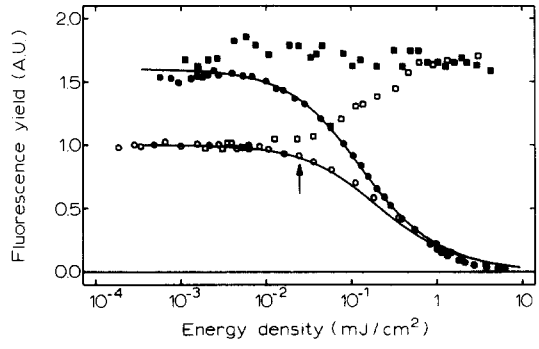

Fig. 2. Annihilation curves of *R. rubrum* chromatophores at 4 K, with reaction centres closed by continuous background illumination (●); reaction centres open before the arrival of the excitation flash (○). In addition the fluorescence intensity caused by a weak xenon flash given 1.5 ms after the laser flash is plotted against the laser intensity (see text). Closed reaction centres (■); reaction centres open before arrival of the actinic flash (□). The fits correspond to the rate constants of Table I. A.U., arbitrary units.

still exists [17]. For the curve with already closed reaction centres (■) the fluorescence yield in the weak xenon flash is independent of the intensity of the preceding laser flash, confirming that the traps are indeed closed. For the curve with the traps initially open (□), the fluorescence yield measured by means of the probing flash increases by a factor of 1.6 at the highest laser intensities. At these intensities the curve tends to become horizontal, which suggests that at these energies the majority of the traps is closed by the actinic flash. However, it should be reminded that at these extreme pulse intensities the competition between annihilation and trapping becomes quite severe, which also may result in the leveling off of the fraction of traps closed by the laser flash [6].

Before turning to a further analysis of the above data, we shall first give a short description of the model of Den Hollander et al. [6], used before by Bakker et al. [13] to analyze their annihilation experiments. This model is based on energy transfer with rate constant k_h between nearest neighbours on a two-dimensional square lattice. The traps, which are regularly spaced on the lattice (N lattice points per trap) can be either open or closed; the corresponding rate constants for trapping are k_t^o or k_t^c, respectively. A domain contains λ traps and N_D ($=\lambda N$) lattice points. The excitations can be lost on each lattice point, with rate constant k_1, and annihilation between two colliding excitations occurs with rate constant k_a. It turns out that the experimentally accessible parameters, fluorescence (or loss) yield and the fraction of traps that are closed can be expressed as functions of the effective or 'averaged' rate constants of trapping, $\bar{k}_t^o$ and $\bar{k}_t^c$ for open and closed traps, respectively, and the average rate of annihilation, $\bar{k}_a$. These average rate constants include the diffusion process the excitation undergoes before trapping or annihilation occurs. From these average rate constants and the size of the domain, the 'molecular' rate constants can then be calculated.

Since, because of a recalibration of the intensity scale, the observed parameters differ somewhat from those found by Bakker et al. [13], we shall first analyze our room temperature experiments. From a loss yield ϕ_l^o of 0.06 when the reaction centres are in the efficiently trapping state P I Q [18], a fluorescence lifetime of 1.0 ns in the absence of traps [19] corresponding to $k_1 = 1 \cdot 10^9$ s^{-1} and from an observed fluorescence increase upon closing the traps of 2.7 at room temperature we obtain, by using the equation

$$\phi_l^{o,c} = \frac{k_1}{k_1 + \bar{k}_t^{o,c}} \tag{3}$$

$\bar{k}_t^o = 1.6 \cdot 10^{10}$ s^{-1} and $\bar{k}_t^c = 5.3 \cdot 10^9$ s^{-1}. From the assumption that $r = 2$ and Eqn. 2 from the introducion it follows that $\bar{k}_a = 6.2 \cdot 10^9$ s^{-1}.

From these effective rate constants the molecular ones can now be calculated, using $N_D = 1000$ and $N = 35$:

$$\frac{\gamma_2}{2} = \frac{\bar{k}_a}{2} = \frac{N_D k_1}{4k_h + k_1}\left[G_{N_D}\left(0; \frac{4k_h}{4k_h + k_1}\right) + \frac{8k_h + 2k_1}{k_a}\right]^{-1} \tag{4}$$

and

$$1 - \phi_l^{o,c} = \frac{Nk_1}{4k_h + k_1}\left[G_N\left(0; \frac{4k_h}{4k_h + k_1}\right) + \frac{4k_h + k_1}{k_t^{o,c}}\right]^{-1} \tag{5}$$

In these expressions, G_{N_D} and G_N are the Green's functions for the domain and the photosynthetic unit, respectively [6]. From Eqns. 4 and 5 the values of k_h, k_t^o and k_t^c can be derived. This yields: $k_h = 1.9 \cdot 10^{12}$ s^{-1}, $k_t^o = 6.1 \cdot 10^{11}$ s^{-1} and $k_t^c = 1.9 \cdot 10^{11}$ s^{-1}. It should be noted that these values are only weakly dependent on the value assigned to r. For $r = 3$ we would obtain a domain size $N_D = 1350$, which yields values of k_h, k_t^o and k_t^c that are smaller by only 8%, $< 1\%$ and $< 1\%$, respectively. The values are summarized in Table I. Although our average rate constants are slightly larger than the ones given by Bakker et al. [13], the molecular values for trapping and energy transfer at room temperature do not significantly deviate from theirs.

Upon cooling from room temperature to 4 K, the fluorescence yield of *R. rubrum* (and therefore the loss yield) increases about four times, in agreement with Rijgersberg et al. [15], from $\phi_l^o = 0.06$ to $\phi_l^o = 0.24$ at 4 K. In addition, our experiments show that N_D decreases strongly. For the ratio ϕ_l^c/ϕ_l^o Rijgersberg et al. [15] reported a value of 1.5 at 4 K. The experiment of Fig. 2 yielded

TABLE I

CHARACTERISTICS OF THE ANTENNA SYSTEM OF *R. RUBRUM* AT TWO TEMPERATURES

The symbols are defined in the text; the rate constants are expressed in s^{-1}

	298 K	4 K
k_l	$1 \cdot 10^9$	$1 \cdot 10^9$
ϕ_l^o	0.06	0.24
ϕ_l^c	0.16	0.38
r	$\geqq 2$	1
N_D	$\geqq 1000$	150
N	35	35
$\bar{k}_t^o$	$1.6 \cdot 10^{10}$	$3.2 \cdot 10^9$
$\bar{k}_t^c$	$5.3 \cdot 10^9$	$1.6 \cdot 10^9$
$\bar{k}_a$	$\leqq 6.2 \cdot 10^9$	$5.3 \cdot 10^9$
k_h	$\leqq 1.9 \cdot 10^{12}$	$1.8 \cdot 10^{11}$
k_t^o	$\geqq 6.1 \cdot 10^{11}$	$1.4 \cdot 10^{11}$
k_t^c	$\geqq 1.9 \cdot 10^{11}$	$6.4 \cdot 10^{10}$

essentially the same ratio of 1.6 and this number was used for further calculations, with the assumption that all traps are closed by a high intensity laser pulse.

Repeating the above calculation with $\phi_l^o = 0.24$, $\phi_l^c = 0.38$, $k_l = 1 \cdot 10^9$ s^{-1}, $N_D = 150$ and $r = 1$ gives the rate constants that are summarized in Table I, together with the room temperature results.

From Table I it can be concluded that, in addition to a large decrease in domain size, a strong decrease in the rates of energy transfer occurs upon lowering the temperature to 4 K. The rate of transfer of an excitation from one BChl molecules to another (k_h) has decreased by a factor of 10, as has also been suggested by Rijgersberg et al. [15] to explain the increased fluorescence yield of *R. rubrum* chromatophores at 4 K. The rates of trapping by the reaction centres are less strongly affected. For open reaction centres the rate of trapping decreased by a factor of about 4, while for already oxidized reaction centres the decrease is by a factor of about 3.

The rate constants given in Table I were used to calculate the fluorescence yield and the number of traps closed by the excitation flash as a function of the number of excitations generated in a domain (Eqn. 21a and b of Ref. 2). The results are given by the continuous curves in Fig. 2. It can be seen that indeed this choice of the rate constants gives a good fit of the experimental data.

Rhodobacter sphaeroides

Fig. 3 shows the time-integrated fluorescence yield of *Rb. sphaeroides* chromatophores at 4 K, detected both at 912 nm where the maximum of the emission by the B875 complex occurs, and at 880 nm where more than 90% of the emission is due to B800–850. The reaction centres were kept in the oxidized state by applying continuous background illumination during freezing and throughout the experiment. It can be seen that the fluorescence yield quenching from the B875 complex starts at much lower intensities than that of the B800–850 complex. For the curve at 880 nm the value of r was found to be close to 1, and the curve at 912 nm for the B875 emission resulted in an r value of at least 2.

When calculating the sizes of the domains, one has to take into account that the excitations are distributed between the B800–850 and the B875 complex. The fractions of the incident quanta at 532 nm that excited the B800–850 and the B875-reaction centre complexes at 4 K were calculated as follows. From the areas under the absorption peaks in the near-infrared region after deconvolution and assuming equal oscillator strengths for BChl 800, 850 and 875, it was calculated that the

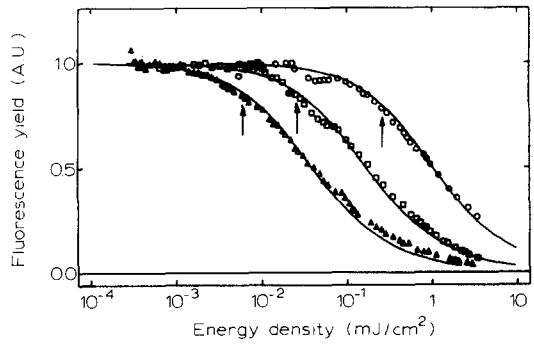

Fig. 3. Annihilation curves of *Rb. sphaeroides* chromatophores at room temperature and at 4 K. The reaction centres were closed by providing the sample with continuous background illumination. 4 K, detected at 880 nm (○); 4 K, detected at 912 nm (□); room temperature, detected at 900 nm (△). A.U., arbitrary units.

ratio of the numbers of BChl *a* molecules in the two complexes in our samples was 3.5:1. On basis of an efficiency of energy transfer from B800–850 to B875 of approx. 90% this means that 78% of quanta absorbed in the Q_x band at 590 nm are used to excite B800–850 and 92% to eventually excite B875 (at low energies). From these numbers, from the absorbance in the band near 590 nm and from the relative heights at 590 and 532 nm in the excitation spectra for the B800–850 and the B875 fluorescence we calculated that for our sample 6.6% of the incident quanta at 532 nm exited B800–850 and 10.6% excited B875. The amount of BChl molecules per unit volume in the sample was, like for *R. rubrum*, calculated from the room temperature spectrum, assuming a shrinkage of the volume of 5% upon cooling. At room temperature, the fraction of absorbed quanta that resulted in excitation of the B875 reaction centre complex was estimated to be 85%.

Using these numbers we calculated the domain sizes at 4 K for the B800–850 and B875 complexes. For the B800–850 annihilation curve (Fig. 3, (○)) the energy was determined where on the average one excitation per domain is generated (with $r = 1$). This, toghether with a BChl 850 extinction coefficient of 184 $mM^{-1} \cdot cm^{-1}$ at 850 nm [20], yielded a domain size of 45 connected BChl 850 molecules. Approximately the same number has been reported for B800–850 complexes, isolated by means of lithium dodecyl sulphate gel electrophoresis [12], indicating that the B800–850 complexes occur in vivo in similar arrangements as in the isolated complex.

The B875 quenching curve reflects not only annihilation in B875 itself, but also annihilation in B800–850, from which energy is transferred to B875. However, from the curves of Fig. 3 it follows that an incident energy at which on the average one excitation per domain of B875 exists, no significant annihilation in B800–850 occurs. Thus we may assume that the yield of energy transfer from B800–850 to B875 is not yet affected at this intensity. We can then perform the same calculations as for the B800–850 complex, and from the low intensity part of the curve we find a domain size of at least 100 (4 K), on the basis of an *r*-value of 2 or more.

The data obtained for *Rb. sphaeroides* can be interpreted by means of the same model we applied above to *R. rubrum*. When the temperature is lowered to 4 K, the loss yield of the B875 complex of *Rb. sphaeroides* with open reaction centres increases by a factor of about 2 from 6% to 12% [15]. With the reaction centres closed, the loss yield increases again by a factor of 2, to 24%. Since the observed ratio of the emission bands of the B850 and the B875 complex is about 1:8, and the initial distribution of the excitations between the two complexes is roughly 50%, the loss yield ϕ'_l of the BChl 850 is about 6%. No reaction centres are present in the B800–850 complex, but the B875 complex can be regarded to act as a trap for the excitations in B800–850. Before a calculation of the various rate constants is possible, we will first have to make an assumption concerning the number of contact sites between the two complexes, since this choice determines the value of N', the number of BChl 850 antenna molecules per B875 trap. The calculations are performed assuming one, two and three contact sites between the B800–850 and the B875 complex. The results are given in Table II, where the rates of transfer of excitations from B850 to the B875 complex are denoted by $\bar{k}'_t$ and k'_t for the average and the molecular rate constant, respectively. The rate constant k_l was again assumed to be $1 \cdot 10^9$ s^{-1}. By using the well-known Förster equation [21]:

$$k_h = \frac{1}{\tau}\left(\frac{R_0}{R}\right)^6 \tag{6}$$

in which τ is the lifetime of a singlet excitation in the absence of nonradiative decay processes, and R_o is proportional to the overlap between the emission band of the donor molecule and the absorption band of the acceptor molecule, we calculate for the distance R between two BChl 850 molecules 1.3 nm, using $R_o = 5.3$ nm, $1/\tau = 5.6 \cdot 10^7 s^{-1}$ [22], and the value of k_h calculated above. In addition, a comparison of the overlap integrals of the BChl 850 emission band with either the BChl 850 or the BChl 875 absorption bands shows that R_o^6 is about 6-times smaller for transfer between two BChl 850 molecules than for transfer from a BChl 850 to a BChl 875 molecule. If we substitute this value, together with the energy transfer rate from BChl 850 to BChl 875, k'_t, into

TABLE II

CHARACTERISTICS OF THE TWO ANTENNA SYSTEMS OF *RB. SPHAEROIDES*

For the B800–850 complex two contact sites with the B875 complex were assumed. One or three contact sites yielded $k_t' = 1.2 \cdot 10^{13}\ s^{-1}$ or $k_t' = 3.1 \cdot 10^{11}\ s^{-1}$, respectively.

	B875 complex		B800–850 complex
	298 K	4 K	4K
k_1	$1 \cdot 10^9$	$1 \cdot 10^9$	k_1 $1 \cdot 10^9$
ϕ_1^o	0.06	0.12	
ϕ_1^c	0.15	0.24	ϕ_1' 0.06
r	$\geqq 2$	$\geqq 2$	r 1
N_D	$\geqq 1000$	$\geqq 100$	N_D 45
N	25	25	N' 23
$\bar{k}_t^o$	$1.6 \cdot 10^{10}$	$7.3 \cdot 10^9$	$\bar{k}_t'$ $1.6 \cdot 10^{10}$
$\bar{k}_t^c$	$5.7 \cdot 10^9$	$3.2 \cdot 10^9$	
$\bar{k}_a$	$\leqq 6.7 \cdot 10^9$	$\leqq 4.2 \cdot 10^9$	$\bar{k}_a'$ $3.3 \cdot 10^{10}$
k_h	$\geqq 2.0 \cdot 10^{12}$	$\leqq 8.7 \cdot 10^{10}$	k_h $2.6 \cdot 10^{11}$
k_t^o	$\geqq 4.2 \cdot 10^{11}$	$\geqq 5.2 \cdot 10^{11}$	k_t' $6.1 \cdot 10^{11}$
k_t^c	$\geqq 1.4 \cdot 10^{11}$	$\geqq 1.1 \cdot 10^{11}$	

Eqn. 6 we obtain the distances between the B850 and the B875 molecules at the contact sites. If one contact site between a B800–850 and a B875 complex is assumed, a distance of 0.92 nm at the contact site is obtained, which appears to be too small to be realistic. With two and three contact sites we obtain values of 1.52 and 1.70 nm, respectively. Thus we conclude that not more than two contact sites between B800–850 and B875 have to be assumed to explain our data.

The data for B875 yield a similar hopping rate as for *R. rubrum*, but 2–4-times higher trapping rates k_t^o and k_t^c (Table II). The room-temperature annihilation curve for BChl 875 emission (Fig. 3, (△)) can be approximately fitted with $r \geqq 2$ and $N_D \geqq 1000$. Since at this temperature a significant rate of energy transfer from BChl 875 to BChl 850 is possible, the domains may consist of several of the B875 domains observed at low temperature, interconnected by B800–850, as will be discussed below. With the restriction that only a poor fit was obtained, and assuming $N = 25$ [23,24] k_h and k_t^o were calculated to be up to $2 \cdot 10^{12}\ s^{-1}$ and at least $4 \cdot 10^{11}\ s^{-1}$, respectively.

At room temperature we obtain the rate of transfer between two BChl 850 molecules from Eqn. 6 and $R_o = 8.2$ nm [22]. This rate then is $1.9 \cdot 10^{12}\ s^{-1}$, which is very close to the value found for the B875 complex of *R. rubrum*. From this room-temperature hopping rate, and assuming that the molecular rate of energy transfer from B850 to B875 does not change upon increasing the temperature, Eqn. 5 then yields an average rate constant $\bar{k}_t^o$ of $2.3 \cdot 10^{10}\ s^{-1}$. This corresponds to a lifetime of an excitation in B850 of about 45 ps, in reasonable agreement with experiments by Sundström and Van Grondelle (unpublished results) who found a lifetime of about 35 ps.

Emission spectra

So far the analysis of the data and their interpretation are based on a model that assumes a homogeneous antenna with traps. Since in this model the emission originates from a single type of antenna molecule, the emission spectrum should not depend on the pulse intensity.

However, it has been proposed by several authors that the long-wavelength B875 antenna of purple bacteria is not homogeneous and might contain an additional pigment absorbing at the long-wavelength side of the main B875 absorption band [25–29]. In order to obtain independent information about the presence of such a pigment and its properties, we measured emission spectra of chromatophores of *R. rubrum* both at low

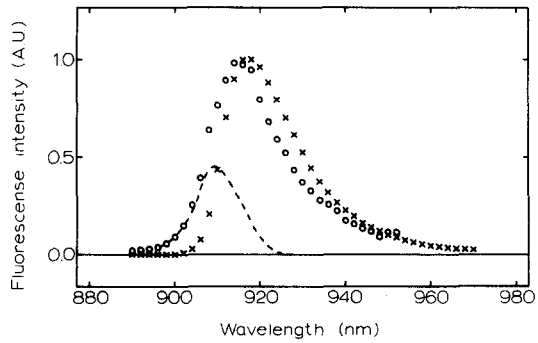

Fig. 4. Emission spectra of chromatophores of *R. rubrum* at 4 K. The reaction centres were closed by continuous background illumination. Excitation at 532 nm by a picosecond laser flash of 4-5 $mJ \cdot cm^{-2}$ (○); excitation by a Xenon flash at 519 nm with an energy of 0.1 $mJ \cdot cm^{-2}$; the spectra are normalized at the emission peak (×); difference spectrum obtained by normalizing the emission spectra at 930 nm and subtracting (○) from (×) (— — —). A.U., arbitrary units.

excitation energy in the absence of singlet–singlet annihilation, and at high pulse intensities where the fluorescence yield has dropped by a factor of 50. The resulting emission spectra are shown in Fig. 4. It can be seen that the spectrum recorded at high excitation intensity is shifted by about 2 nm to shorter wavelengths compared to the low-intensity spectrum. At room temperature no significant difference between the emission spectra obtained at high- and low-excitation energy could be detected, as had also been noticed by Bakker et al. [13]. The effect of a long-wave pigment on the emission spectrum should indeed be small at room temperature, since then the excitations can move freely from one type of pigment to another and vice versa. At 4 K, however, the rate of back transfer of excitations which have arrived in the long-wave pigment would be small.

If we make the assumption that the spectrum obtained at 4 K at high intensity is a composite spectrum containing a contribution from a long wavelength component, called BChl 896 by Kramer et al. [27], and a short wavelength main component BChl 875, and if we take for the BChl 896 emission the spectrum obtained at low excitation intensities [27], we can estimate the shape of the additional contribution to the emission spectrum at high pulse intensities. This is also shown in Fig. 4 and it can be concluded that the additional emission spectrum peaks at about 909 nm, i.e., 8 nm shorter wavelength than the main emission. It thus appears that at high pulse intensities the 'bulk' BChl 875 contribution to the total emission becomes significant. This indicates that at low light intensities the diffusion of the excitation among the BChl 875 molecules is sufficiently efficient to trap the excitation either on BChl 896, from which the fluorescence occurs, or on the reaction centres. At high pulse intensities the diffusion of the excitations becomes severely limited due to excitation annihilation, giving rise to a relative decline of the BChl 896 emission and of the trapping efficiency.

It is interesting to note that similar spectral effects have not been observed in chloroplast fluorescence [30]. However, recent experiments [31] indicate that very high intensities are needed to observe annihilation in long-wave emitting chlorophylls, and possible effects of this annihilation on the emission spectrum in the far-red region have not yet been investigated.

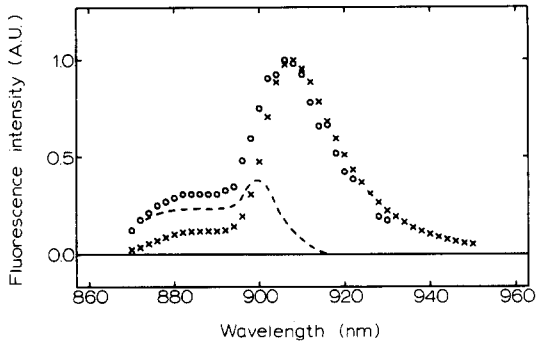

Fig. 5. Normalized emission spectra of *Rb. sphaeroides* chromatophores at 4 K, obtained at high (○) and low (×) intensity exciting flashes as in Fig. 4. Difference spectrum obtained by normalizing the spectra at 920 nm (— — —). A.U., arbitrary units.

In Fig. 5 we show emission spectra of chromatophores of *Rb. sphaeroides* at 4 K. Again we observe that the emission peak shifts to shorter wavelengths upon increasing the energy of the excitation flash. In addition to this, the shape of the spectra of Fig. 5 is consistent with the observation shown in Fig. 3, that the fluorescence yield of B800–850 does not drop as quickly at high laser-pulse intensities as the B875 fluorescence yield. While at low excitation energies the B800–850 emission only appears as a weak shoulder, at high energies the relative contribution of the B800–850 emission increases by approx. a factor of 3.

Discussion

The results presented here show clearly that upon lowering the temperature from 298 to 4 K in *R. rubrum* as well as in *Rb. sphaeroides* a strong decrease occurs in the sizes of the domains for energy transfer. An analysis for *R. rubrum*, which has a simpler antenna structure than *Rb. sphaeroides* indicates that the rate of energy transfer between the BChl 875 antenna molecules (k_h) decreases about 8-fold. In addition the trapping rates for closed and open reaction centres k_t^c and k_t^o are reduced by a factor of 3–4.

From measurements of fluorescence lifetimes, Freiberg et al. [32] recently concluded that in

isolated reaction centres of *R. rubrum* the rate of primary electron transfer is 7 ± 2 ps, independent of the temperature. This indicates that the trapping rates k_t° obtained by us (Table I), corresponding to trapping times of 1.6 ps at room temperature and of 7 ps at 4 K, at least partly reflect the energy transfer from the antenna to P, rather than the charge separation alone (see below).

Rijgersberg et al. [15] already suggested that the rate of energy transfer between antenna pigments would be slower at 4 K by about a factor of 10. Our results give independent support for this suggestion. Because the rates of trapping do not decrease as strongly as the rate of energy transfer between antenna molecules, this implies that the increase in fluorescence yield that occurs upon lowering the temperature is only partly due to a decreased efficiency of the traps, and to a much larger degree due to a slower energy transfer between antenna pigments.

Both at room and low temperature, the hopping rates k_h are higher than the rates of trapping k_t°. This would imply that an exitation on the trap or on a lattice point next to the trap has a higher probability to escape and to continue its random walk in the antenna than to be trapped. This is in agreement with results of Freiberg et al. [33] and of Bakker et al. [13], but appears to be in contrast to the observation that upon excitation of reaction centre bands no fluorescence is emitted by membranes of *R. rubrum* and *Rb. sphaeroides* [34] and of the green gliding bacterium *Chloroflexus aurantiacus* [35] when the reaction centre traps are open. For a discussion of this point we refer to Ref. 1.

Not only the various rate constants are smaller at low temperature, but also the sizes of the domains involved in energy transfer. While at room temperature the number of connected antenna molecules in *R. rubrum* is approximately equal to the number of antenna pigments present in a single chromatophore (about 1000) [36], at 4 K the domain breaks into smaller parts containing on the average only 150 antenna molecules, connecting four reaction centres. Recently, Hunter et al. [37] observed that in a developing photosynthetic membrane at least four reaction centres were connected, even in the earliest stage of development. This result, together with our observations, suggests that the antenna is synthesized in clusters of four reaction centres. These 'minimal units' then assemble into larger domains when the photosynthetic apparatus of the bacterium further develops. There will, however, still remain some barriers between the different clusters during this maturation process. These barriers are obviously easy to overcome for excitations at room temperature, but at 4 K they become difficult to cross.

A similar picture applies for *Rb. sphaeroides*. In this bacterium, at 4 K small domains of B875 exist, again connecting about four reaction centres. The B800–850 complexes also form small domains, of 45 BChl 850 molecules, which transfer their energy to B875 perhaps via only a few contact sites. This indicates that at low temperature the model proposed by Monger and Parson [10], developed from room temperature experiments on triplet quenching, does not apply. In this model the B800–850 complex forms a 'lake' around large clusters of B875, which interconnect the reaction centres. Since the ratio BChl 875 to BChl 850 in our preparation was 1:2.3, there are about 230 BChl 850 molecules per B875 domain, corresponding to about five B800–850 domains. This leads us to the proposed structure of the antenna of *Rb. sphaeroides* at 4 K shown in Fig. 6.

In our analysis thus far, the presence of a long-wave absorbing BChl (BChl 896) in B875 has not been taken into account. As discussed above, the observed blue shift of the emission bands when pulses of high intensity are used to excite the sample can be interpreted assuming the presence of such a pigment. The effect of BChl 896 on the excitation annihilation and trapping processes is not easy to estimate. At room temperature its presence may, in first order, be ignored, since most of the excitations will be localized on B875. However, at 4 K this will, if the Boltzmann distribution would be established sufficiently rapidly, not be the case and we might expect a strong effect. Nevertheless, a model which ignores the presence of BChl 896 leads to a good fit of the experimental results. If we suppose, for instance, that in *R. rubrum* about 25 BChl 896 pigments are more or less randomly distributed over a domain of 150 BChl molecules, each of them would probably act as a relatively strong trap. Energy transfer

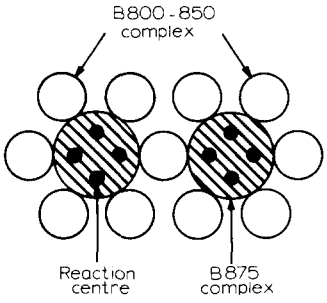

Fig. 6. Model of the antenna structure of *Rb. sphaeroides*. The areas of the circles correspond to the domain sizes of the B800–850 and the B875 complex, observed at 4 K. Since this figure probably represents half of the total amount of pigment present in a single chromatophore, no attempt has been made to organize the complexes in a more regular lattice.

to the reaction centre seems unlikely in that case. Moreover, if two excitations are present in the domain they probably would be localized rapidly on two different BChl 896 molecules and would have a small probability to interact. In that case the 4 K annihilation curve would show extremely inefficient annihilation (large r values) contrary to what we observed.

In view of these arguments we propose the following: (1) BChl 896 represents a cluster of long wavelength antenna molecules, in contact with the reaction centre. (2) The trapping of excitation energy and the annihilation of excitations are mainly governed by the transfer of excitations in the B875 antenna. Annihilation may occur between two freely diffusing excitations or one diffusing excitation and one that is localized on BChl 896. It is important to remark here that in the random walk description of the annihilation process in first approximation it is immaterial whether both excitations diffuse or only one does while the other is localized [6]. (3) Upon arrival in BChl 896, the excitation may be lost or transferred to the reaction centre.

It is difficult to establish the meaning of the uniform hopping-rate constant k_h calculated in Table I. From the above it is obvious that it will contain contributions from BChl 875 to BChl 875 and from BChl 875 to BChl 896 transfer. However, it is clear that a discussion on the role of BChl 896 remains speculative unless supported for instance by a computer simulation of the process that occurs at 4 K. In this connection also experiments with *Rhodopseudomonas viridis* might be of interest, since in this species the long-wave pigment does not appear to be present [38].

Acknowledgements

The authors are indebted to Dr. M.J.M. van de Ven for valuable assistance. We also want to thank R.J. van Dorssen for his help with the measurement of excitation spectra, and M. van Dijk for measuring absorbance difference spectra. The investigation was supported by the Netherlands Foundation for Biophysics, financed by the Netherlands Organization for the Advancement of Pure Research (ZWO).

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