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Singlet–singlet excitation annihilation measurements on the antenna of *Rhodospirillum rubrum* between 300 and 4 K

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By means of fluorescence measurements singlet–singlet excitation annihilation in the antenna of *Rhodospirillum rubrum* chromatophores was studied at temperatures between 300 and 4 K. Two fluorescence bands had to be assumed to explain the data at 4 K. These bands (F911 and F918) are attributed to emission from the main antenna pigment BChl 880 and from a minor spectral component BChl 896, respectively. The latter pigment is supposed to be associated with the reaction center (Van Grondelle, R., Bergström, H., Sundström, V., Van Dorssen, R.J., Vos, M. and Hunter, C.N. (1988) in Photosynthetic Light-Harvesting Systems (Scheer, H. and Schneider, S., eds.), pp. 519–530, Walter de Gruyter, Berlin). At 4 K 90% of the fluorescence originates from this pigment. Analysis of the data at 4 K indicated that BChl 896 is arranged in clusters of 21 ± 9 BChls. Since there are presumably only 5 or 6 BChls 896 per reaction center, this indicates that several reaction centers are clustered. Annihilation measurements on the isolated B880 antenna complex indicate that, at 4 K, the spectral heterogeneity still exists and that the presence of BChl 896 is therefore not caused by interactions between the reaction center and the antenna. The domain size of BChl 880 was estimated to be between 35 and 75 BChls in chromatophores at 4 K. A wavelength dependence of the annihilation, due to the presence of the two types of BChl, was observed between about 100 and 4 K. Measurements of fluorescence polarization suggest that this wavelength dependence may be caused by a reduction of the rate of energy transfer from BChl 896 to BChl 880 upon cooling. An increased annihilation between 100 and 300 K can, at least partly, be ascribed to an enhanced rate of energy transfer between individual BChls. This rate increases from an average value of $(4 \pm 1) \cdot 10^{11}$ s^{-1} at 100 K to $(1.5 \pm 0.5) \cdot 10^{12}$ s^{-1} at 300 K.

Introduction

Photosynthetic reaction centers receive their energy from surrounding pigment molecules [1], which serve to increase the rate of the photosynthetic process. The assembly of a reaction center with surrounding pigments is called a photosynthetic unit. Often photosynthetic units are interconnected and form large aggregates. Within these larger structures, which are called domains [2], excitations can be transferred from one molecule to another until a reaction center is reached.

There are only few experimental methods that can give insight into the sizes and topology of the domains. The method we employed is the detection of the fluorescence yield of the antenna pigments as a function of the intensity of a laser excitation pulse. At high pulse energies several excitations may be generated in one domain. Energy transfer within the domain may result in the formation of higher excited states [3] by two excitations accumulating at one pigment molecule. Rapid decay by radiationless processes to the lowest excited state then causes a decrease of the fluorescence yield. This effect is called singlet–singlet excitation annihilation.

A relation between the fluorescence yield ϕ and the number of excitations generated per domain (z) was given by Paillotin et al. [4]:

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

where $\phi(0)$ is the fluorescence yield at low excitation

Abbreviations: BChl, bacteriochlorophyll; BChl 880 and 896, bacteriochlorophyll absorbing around 880 and 896 nm, respectively; B880, light-harvesting complex of *Rhodospirillum rubrum* containing BChl 880 and BChl 896; F911 and F918, bacteriochlorophyll fluorescence bands with maxima near 911 and 918 nm, respectively.

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intensity where no annihilation occurs. The parameter r is equal to twice the ratio of the averaged rate of mono-excitation decay (γ_1) and the rate of annihilation (γ_2) per pair of excitations. An important assumption in the derivation of Eqn. 1 is that the excitation distribution is random at all times.

The experimental data may be analyzed by fitting Eqn. 1 for various values of r to the measurements. This results in a value for the energy density for which $z = 1$. For this energy density the number of excitations initially present in the sample can be calculated and, as $z = 1$ at this density, also the number of domains in the sample is known. Then the domain size is calculated from the total number of BChls in the sample.

The method has been applied successfully by Bakker et al. [5] and Vos et al.[6] in studies of the antennae of *Rhodospirillum rubrum* and *Rhodobacter sphaeroides*. At room temperature domain sizes of at least 1000 BChls in *R. rubrum* were obtained [6]. At 4 K less annihilation was observed and it was concluded that the domain size was reduced to about 150 BChls. At low temperature Vos et al. [6] also observed a shift of the fluorescence spectrum as the intensity of the laser excitation pulse increases, indicating that the annihilation process is wavelength dependent.

In this paper we report a more detailed investigation of excitation annihilation in *R. rubrum*, as a function of both temperature and wavelength. We further studied a B880 antenna complex of *R. rubrum* from which the reaction center had been removed. Our results can be explained by the assumption that spectral inhomogeneities exist in the antenna of *R. rubrum* at low temperatures. These inhomogeneities may be attributed to the presence of a long-wavelength absorbing pigment fluorescing at 918 nm at 4 K in addition to the main antenna BChl 880, which fluoresces at 911 nm. In the literature the former pigment is often referred to as the minor spectral component or BChl 896 [7–10]. We also studied the spectral inhomogeneity by means of fluorescence polarization measurements. Our results indicate that the temperature dependence of the annihilation characteristics is largely determined by the excitation transfer rate from BChl 896 to BChl 880.

Materials and Methods

R. rubrum strain S1 was cultured in the medium of Cohen-Bazire et al. [11]. Chromatophores were prepared by sonifying the cells for 10 min at 0 °C, followed by high-speed centrifugation. The chromatophores were suspended in a buffer containing 10 mM Tris (pH 8.0), 5 mM $MgCl_2$ and 0.5 M sucrose.

R. rubrum B880 antenna complexes with at least a 10-fold reduced reaction center content were a kind gift of Dr. E.A. Kotova of the M.V. Lomonosov Moscow State University. They were prepared from *R. rubrum* chromatophores by solubilization with lauryldimethylamine *N*-oxide according to the procedure described by Drachev et al. [12]. They were suspended in the same buffer as the chromatophores. Glycerol at 65% (v/v) was added to all samples to obtain a transparent glass at low temperatures.

The spectrofluorimeter used for the annihilation experiments was described elsewhere [5]. The wavelength of the exciting laser pulse was 532 nm. The maximum energy in the pulse was 5 mJ/cm^2 and the pulse length was approx. 25 ps. Low-intensity emission spectra were measured with xenon flash illumination through a Schott Al-522 interference filter. For measurements between 4 and 300 K a helium flow cryostat (Kelltor) was used. All measurements on *R. rubrum* chromatophores were performed with the primary donor in the oxidized state (P^+). In order to obtain this state the sample was illuminated during cooling and throughout the experiment. The redox state of the reaction center was checked by measuring the fluorescence yield due to excitation with a weak xenon flash. The absorbance of the samples at 532 nm was kept low (less than 0.1) in order to achieve a homogeneous light distribution within the sample. We observed that photodegradation of the samples led to a decrease of the fluorescence yield and an increase of mono-excitation relative to bi-excitation decay processes. Fresh samples were therefore used after each series of measurements.

Both the excitation energy and the fluorescence yield were measured with photodiodes (RCA C30810). The fluorescence yield was compared by a least-squares analysis with computer-generated curves of Eqn. 1. The data were then analyzed as described by Vos et al. [6]. In general, the spread of the data was such that fits of Eqn. 1 to the data could not be distinguished for $r \geqq 2$. We assumed that 30% of the energy absorbed at 532 nm, at all temperatures, is transferred to the antenna BChl [13].

Temperature-dependent fluorescence polarization experiments were performed using a single-beam spectrophotometer as described by Kramer et al. [8].

Results and Discussion

The relative fluorescence yield as a function of the laser pulse energy for *R. rubrum* chromatophores is shown in Fig. 1. The solid lines are fits of Eqn. 1 to the data and will further be called annihilation curves. The measurements were performed at 4 K, at two different emission wavelengths. At both wavelengths a decrease of the fluorescence yield is observed as the laser excitation energy increases. This decrease is caused by singlet–singlet annihilation. Analysis of the measurements according to the procedure described above (see Materials and Methods) leads to an apparent domain size of 220 ± 50 BChls and an r-value of 0.6 ± 0.2 for the

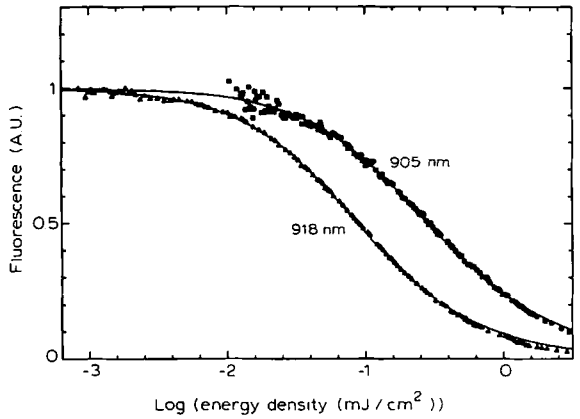

Fig. 1. The relative yield of fluorescence plotted as a function of the incident energy density of the excitation flash for *R. rubrum* chromatophores at 4 K. Squares: detected at 905 nm; triangles: detected at 918 nm. Excitation was at 532 nm with a 25 ps laser flash. The data are fitted with Eqn. 1 with $r = 1.5$ (905 nm) and $r = 0.6$ (918 nm). The curves are normalized at low energy densities. A.U., arbitrary units.

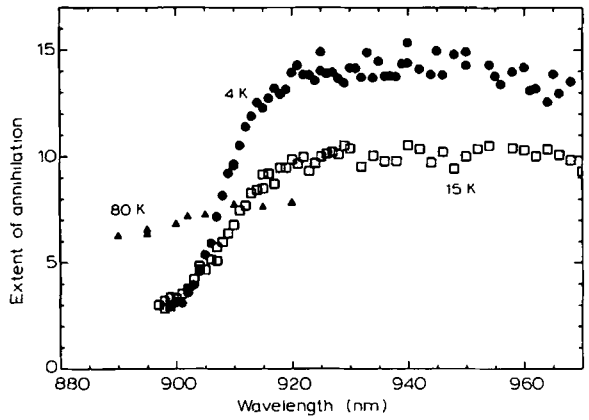

Fig. 3. Wavelength dependence of the extent of annihilation (see text) at 4 K (circles), 15 K (squares) and 80 K (triangles).

annihilation curve at 918 nm. A similar result was obtained by Vos et al. [6]. For the curve at 905 nm an apparent domain size of 120 ± 25 and an r-value of 1.5 ± 0.4 is obtained. Analyzing the data at different wavelengths therefore leads to different domain sizes and r-values. This can only be explained if it is assumed that the antenna of *R. rubrum* is spectrally inhomogeneous.

In order to obtain information about the extent of annihilation quenching as a function of wavelength, the fluorescence spectrum of *R. rubrum* was recorded at high and at low pulse energies. The low intensity spectrum (Fig. 2a, solid line) and the high intensity spectrum (Fig. 2b, solid line) were recorded on the same spectrophotometer with xenon flash and with laser pulse excitation, respectively. The applied laser pulse energy density was 1.0 mJ/cm^2. The extent of annihilation at high light intensity as a function of emission wavelength, obtained by dividing the first by the second spectrum, is shown in Fig. 3 (solid circles). It can be seen that the extent of annihilation increases with wavelength until it reaches a constant level at 920 nm, indicating that the fluorescence above 920 nm comes from one type of pigment. Assuming that the fluorescence spectrum consists of two bands, the extent of annihilation at each wavelength is a combination of the contribution by these bands. The data of Fig. 3 can then be used to calculate the sizes and shapes of the contributing fluorescence bands. The two calculated fluorescence bands are shown in Fig. 2 by the broken lines. One band has a maximum at 918 nm with a width at half maximum of 21 nm. Since the annihilation behavior over this band is constant at 4 K, we conclude that this band is due to a single pigment. The other has a maximum at 911 nm with a width of 11 nm. These bands, which we will call F918 and F911, presumably arise from BChl 896 and BChl 880, respectively. The ratio of the integrated fluorescence yields at low intensity of the two bands is 11.7 ± 0.8. Results of measurements at 15 K and 80 K are also plotted in Fig. 3. It is observed in Fig. 3 that upon lowering the temperature from 80 to 4 K, there is an increase of the extent of annihilation at wavelengths longer than 920 nm, from about 8 to about 14 at the energy density applied. At 15

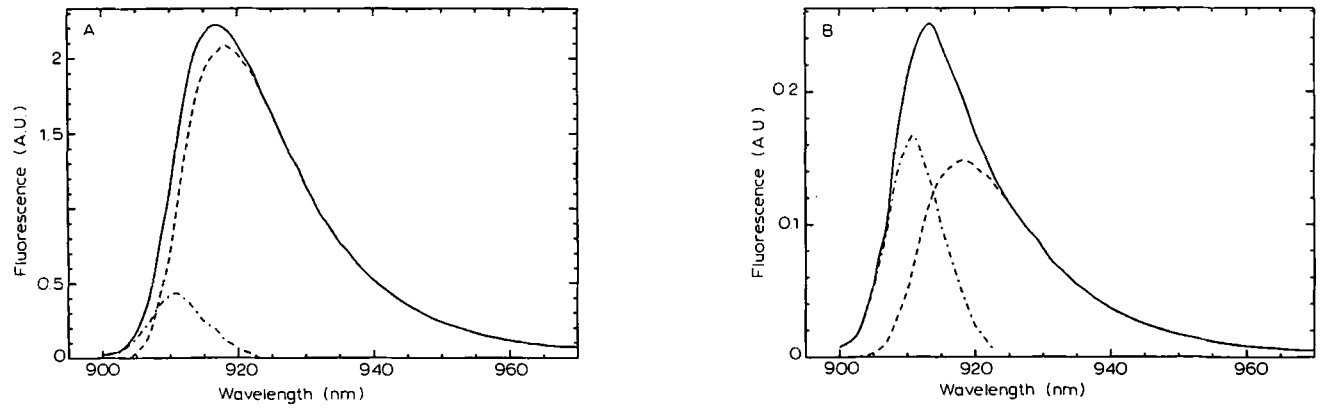

Fig. 2. (A) Fluorescence spectrum at low excitation intensity fluorescence spectrum of *R. rubrum* chromatophores at 4 K (solid line) and decomposition in two bands (broken lines). (B) As (A), but measured at high excitation intensity (1.0 mJ/cm^2).

K the calculated positions and widths of the two bands were the same as at 4 K with a ratio of 9.7 ± 1.2 at low excitation intensity. At 80 K the wavelength dependence had almost disappeared.

Since the antenna of *R. rubrum* is spectrally inhomogeneous at low temperatures, Eqn. 1 is not in a simple way applicable to this system. When analyzing the fluorescence of F911, the pigments emitting at 918 nm can be considered to act as traps and their presence is therefore reflected by the mono-excitation decay γ_1. As noted by Kramer et al. [8], the high polarization value of the fluorescence indicates that back transfer from BChl 896 to BChl 880 does not take place at 4 K. Thus, in principle, Eqn. 1 remains applicable to F911. A correction, however, must be made for the fact that the curve recorded at 905 nm is not entirely due to F911. At low excitation intensity about 25% of the fluorescence yield originates from F918 (see Fig. 2). Applying the correction for the contribution of F918 at 905 nm an *r*-value of 1.0 ± 0.3 and a domain size of 75 ± 25 BChls were obtained. However, for BChl 880 a correction must also be made for the pulse duration of 25 ps, which is of the same order of magnitude as the lifetime of F911. Measurements of fluorescence lifetimes [14] and of absorption recovery [15] indicate that at 77 K the lifetimes of F911 and F918 with closed reaction centers are approx. 20 and 160 ps, respectively. We observed an increase of the total fluorescence yield by a factor of 2.5–3 when lowering the temperature from 77 to 4 K. From this number and a ratio of the integrated fluorescence intensities of the two bands of 10 at 4 K, we thus calculated a lifetime at 4 K of 50 ± 10 ps for F911 and of 500 ± 100 ps at 4 K for F918, respectively.

To study the effect of the length of the laser pulse we performed random walk computer simulations on a square lattice of 36 molecules similar to the ones performed by Vos [16]. For these simulations we assumed that the excited BChls decay with a time constant of 50 ps. The time needed for energy transfer between individual molecules was taken to be 1.5 ps. Constant amplitude pulses of 1, 25 and 50 ps were used. The annihilation curves thus obtained are shown in Fig. 4. The random walk simulations were fitted with curves calculated according to Eqn. 1 by which *r*-values were determined, as well as the number of excitations for which $z = 1$. The *r*-value increased from $r = 0.5$ for the 1-ps pulse to $r = 1.3$, as in the experiment, for the 25-ps pulse. The number of excitations for which $z = 1$ is shown in Fig. 4 by markers on the horizontal scale. For the 1-ps pulse this value corresponds to 1 true excitation on the lattice. For the pulse with 25 ps duration $z = 1$ corresponds to 0.75 true excitations on the lattice. This means that applying a 25-ps pulse the domain size will be overestimated by about a factor of 1.3. Assuming that this factor is also valid for our experiment, the obtained domain size of 75 ± 25 BChls should be divided by this factor. We thus determined a domain size of 55 ± 20 BChls 880.

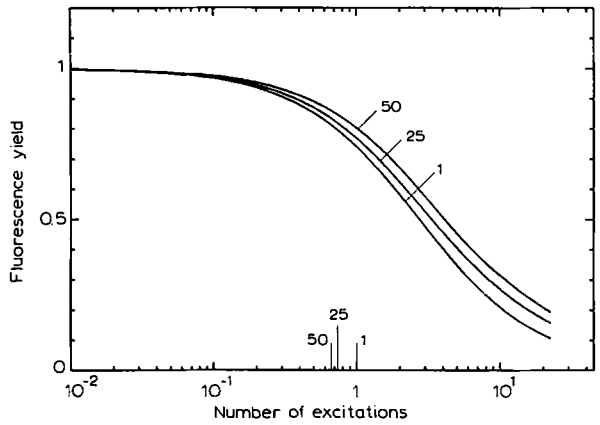

Fig. 4. Simulated annihilation curves for a 6×6 lattice for laser pulse lengths of 1, 25 and 50 ps, respectively (see text). The vertical lines give the corresponding intensity for which the (apparent) *z*-values are equal to unity. In the simulation annihilation is assumed to occur if two excitations are present on the same BChl.

Concerning the F918 fluorescence component, we note that most of the excitations in the corresponding BChl 896 will result from energy transfer from BChl 880. Therefore the number of excitations present in BChl 896 has to be corrected for annihilation in BChl 880. In fact the annihilation curve of F911 determines the relative amount of energy transferred to BChl 896 and therefore the excitation density in BCl 896. On the assumption that this pigment constitutes about 15% of the total antenna as in *Rhodobacter sphaeroides* [8] and receives 85% of the excitations via BChl 880, an *r*-value of 0.5 ± 0.4 and a domain size of 21 ± 9 are found. The relatively large error in these values is mainly caused by the fact that the *r*-curves fit the data poorly at high energies. The curves were therefore only fitted up to a density of 5 excitations per domain. The relatively large domain size found for F918 is interesting. Since probably only 5–7 BChls 896 are present per reaction center [8], this indicates that each reaction center is closely interconnected with 1–4 others. It is of interest to note here that measurements by Hunter et al. [17] on developing photosynthetic membranes revealed that the minimal functional size in the chromatophore of *R. rubrum* is about 4 photosynthetic units large. The assumption used above that BChl 896 constitutes 15% of the total antenna [8] is based on the assumption that the extinction coefficients of BChl 896 and BChl 880 are the same. If, for example, in the red the extinction coefficient is higher for BChl 896, the number of BChl 896 in the sample will be overestimated. The number of domains, however, derived from the annihilation curves, is independent of the relative absorption of BChl 880 and BChl 896. In this case the calculated domain size of BChl 896 will be too large. However, since then the calculated number of BChl 896 per reaction center is

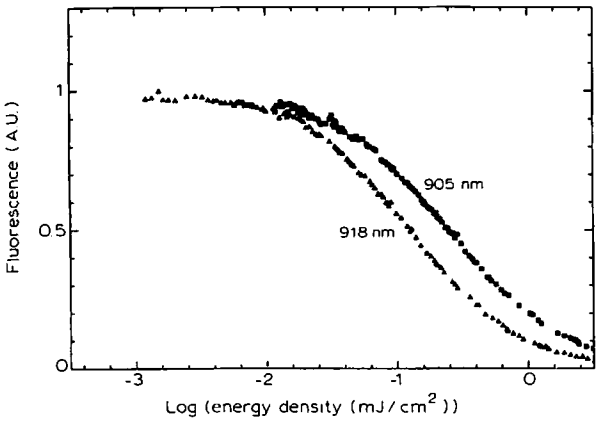

Fig. 5. Energy density dependence of the fluorescence for the isolated B880 complex of *R. rubrum*, detected at 905 (squares) and 918 nm (triangles). The data are normalized at low energy densities.

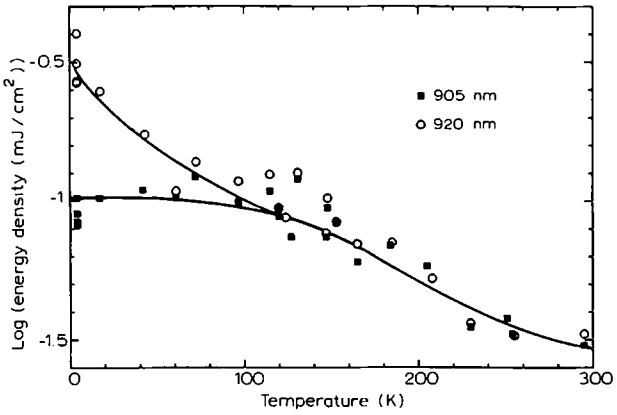

Fig. 6. Temperature dependence of the energy density for which the relative fluorescence yield has decreased to 50% for *R. rubrum* chromatophores. Detected at 905 (squares) and 920 nm (circles), respectively.

also overestimated, the number of interconnected reaction centers is independent of the relative absorption in the red.

Annihilation measurements performed at 4 K on the isolated B880 antenna complex are shown in Fig. 5. The measurements show a similar wavelength dependence of the annihilation curves as the chromatophores of *R. rubrum*. This indicates that the pigment fluorescing at 918 nm is still present in the complex. This was also shown by picosecond absorption difference spectroscopy by Danielius et al. [18] at room temperature. In view of the similarity between the data on chromatophores and those on the antenna complex we presume that the domain sizes for both systems are roughly the same. Since the results indicate that BChl 896 is still present in antenna complexes of *R. rubrum* devoid of reaction centers, we conclude that the spectral properties of BChl 896 result from local interactions within the antenna, rather than from an interaction between the reaction center and the antenna BChls.

To study the temperature dependence of annihilation in *R. rubrum* chromatophores in more detail, we measured annihilation curves between 4 and 300 K at 905 and 920 nm. The results are shown in Fig. 6. In this figure the energy density is plotted for which the fluorescence yield has decreased to 50%, relative to the value obtained in the absence of annihilation, as a function of the temperature. Fig. 6 shows that there is a wavelength dependence of the energy density at which 50% annihilation occurs, observable to about 100 K. Upon increasing the temperature from 100 to 300 K there is a shift of the 50% annihilation point towards lower densities. Annihilation curves at temperatures between 25 and 300 K could, at both wavelengths, be fitted with $r \geqq 2$. At lower temperatures the r-value dropped from about 2 at 25 K to 0.6 at 920 nm and to 1.5 at 905 nm at 4 K.

There are probably several factors that contribute to the temperature dependence of the annihilation. One of these is a decrease of the rate of back transfer between BChl 896 and BChl 880 with decreasing temperature. This effect can also be observed in fluorescence polarization measurements as a function of temperature. Fig. 7 shows the polarization of fluorescence (p) at 940 nm upon excitation in the absorption band of BChl 896. At 4–20 K a strong polarization with an average value of 0.39 was obtained. This number is in close agreement with earlier results at 4 K and has been explained by the absence of back transfer from BChl 896 to BChl 880 and a high degree of orientation of BChl 896 [8]. Upon increasing the temperature the polarization drops, reaching a value of 0.18 at 80–100 K and of 0.13 at 300 K, presumably reflecting both an enhanced rate of back transfer, and increased overlap of the absorption bands of BChl 880 and BChl 896.

We performed random walk simulations to study qualitatively the effect of the back-transfer rate on the annihilation. A square lattice of 400 BChls was used. 54 BChls (14%) represented a long-wavelength absorbing species, which were distributed in nine groups of six BChls. The group and lattice size are both smaller than

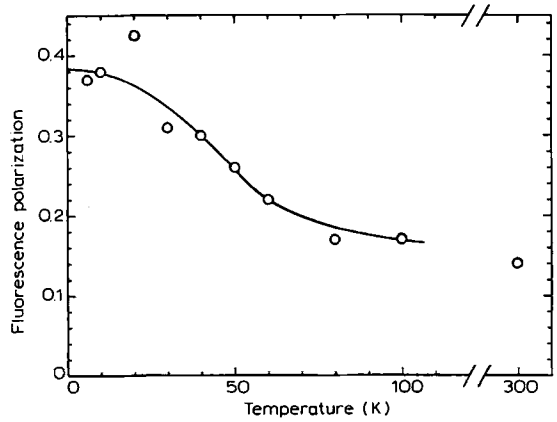

Fig. 7. Temperature dependence of the fluorescence polarization, detected at 940 nm with excitation at 915 nm.

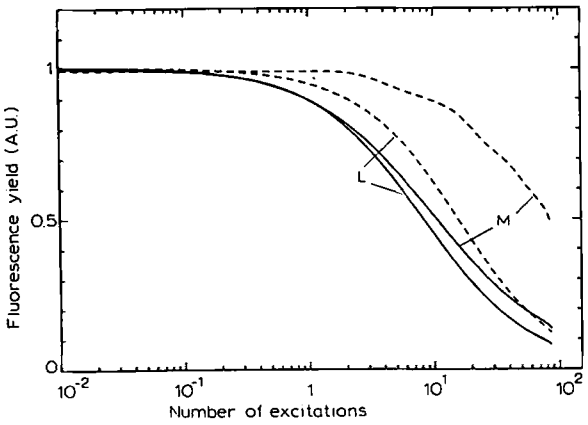

Fig. 8. Simulated annihilation curves for a 20×20 lattice consisting of 346 main antenna BChls and nine regularly spaced clusters of six long-wavelength absorbing BChls with different rates of back transfer k_b; broken lines: $k_b = 0$; solid lines: $k_b = 1 \cdot 10^{10}$ s^{-1}. The curves marked M refer to emission from the main BChl, those marked L to the long-wavelength-absorbing BChl. The other rate constants are given in the text. An excitation pulse of 25 ps duration and constant amplitude was used. Other details are given in the text.

the experimentally obtained domain sizes of BChl 896 at 4 K and BChl 880 at 300 K, in order to limit the CPU time needed. The energy-transfer rate between two identical BChls was taken to be $1 \cdot 10^{11}$ s^{-1}. It was assumed that this rate is slightly higher ($1.5 \cdot 10^{11}$ s^{-1}) if transfer takes place from a main antenna BChl to a long-wavelength absorbing BChl. The loss rate due to fluorescence was taken to be $1 \cdot 10^{9}$ s^{-1}. To simulate a temperature dependence of the annihilation only the back transfer rate (k_b) was varied. For simplicity, all other rates were assumed to be temperature-independent. In Fig. 8 annihilation curves are shown for $k_b = 0$ (dashed lines) and $k_b = 1 \cdot 10^{10}$ s^{-1} (solid lines) for the main antenna BChl (M) and the long-wavelength-absorbing BChl (L). The figure shows that if back transfer is absent ($k_b = 0$) a strong wavelength dependent is observed, but if the back transfer rate is 1/15 of the forward transfer rate ($k_b = 1 \cdot 10^{10}$ s^{-1}) the wavelength dependence is small and will be difficult to resolve experimentally. The BChl 896 $S_0 \rightarrow S_1$ transition occurs at lower energy than that of BChl 880, so that in first approximation it may be assumed that back transfer can be described by an Arrhenius law:

$$k_b = k_f e^{-\Delta E/kT} \qquad (2)$$

where the activation energy ΔE corresponds to a 10 nm difference of the absorption maxima of the pigments involved. If we further assume that the back-transfer rate at high temperature is equal to the forward rate k_f, then $k_b = 1 \cdot 10^{10}$ s^{-1} corresponds to a temperature of about 70 K. Experimentally a wavelength dependence therefore will only be observed at lower temperatures.

If we analyze the results of the simulation for $k_b = 0$ (dashed lines) by means of Eqn. 1, using the same corrections as applied for chromatophores, we obtain a domain size for the long wavelength absorbing pigment of 6 BChls, which is equal to the actual number. This is an indication that Eqn. 1 remains applicable also for heterogeneous systems. For the main antenna pigment a domain size of at least 30 BChls was obtained.

Between 100 and 300 K the 50% annihilation point showed a considerable shift towards lower energy densities with increasing temperature. No wavelength dependence of the annihilation was detectable at these temperatures, so we assumed that the annihilation in the antenna can be approximated by a homogeneous model. Since only a lower limit for r ($r \geqq 2$) could be determined from an analysis of the annihilation curves, only lower limits for the corresponding domain sizes can be calculated: at least 300 BChls at 100 K and at least 1000 BChls at 300 K. For this reason it is not clear whether the size of the domains changes in the temperature region above 100 K. In spite of this uncertainty, it can be shown that a considerable change in the average rate of energy transfer between individual BChl molecules must occur in this temperature region. The upper limit for the domain size can be taken as 10000 BChls, the maximum number of BChls in a chromatophore [19]. This means that at room temperature $2 \leqq r \leqq 50$. Calculation of the corresponding average energy-transfer rates (k_h) according to the model of Den Hollander et al. [20] then gives $1.0 \cdot 10^{12}$ s$^{-1} \leqq k_h \leqq 2.0 \cdot 10^{12}$ s^{-1}. At 100 K these numbers are $3.0 \cdot 10^{11}$ s$^{-1} \leqq k_h \leqq 5.0 \cdot 10^{11}$ s^{-1}. Our calculations show that these values do not critically depend on the precise r-values and domain sizes, but are mainly determined by the 50% points as given in Fig. 6, because a larger r-value and a corresponding larger domain to some extent cancel each other when calculating the energy-transfer rate.

As discussed above, the decrease in annihilation observed when cooling down from 300 K to about 100 K

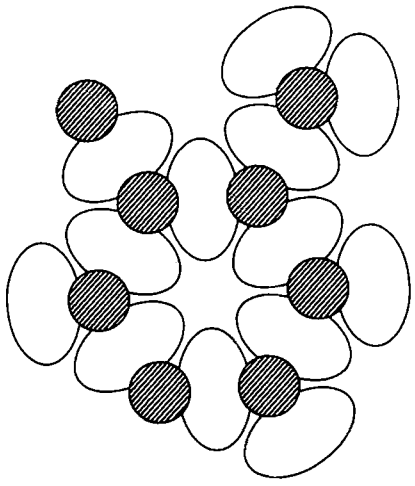

Fig. 9. Possible arrangement of BChl 880 ellipses) and BChl 896 (hatched circles) in the antenna of *R. rubrum*. The ellipses and the circles represent approx. 60 and 20 BChls, respectively.

does not necessarily reflect a decrease in domain size and can be explained solely by a decrease in the average rate of energy transfer between BChls, caused by a decrease in the Förster overlap integral. Nevertheless, it is clear that upon further cooling to liquid helium temperatures the domains are broken up in relatively small clusters of BChl 880 and BChl 896. It is likely that the decrease in domain size of BChl 880 by at least a factor of 4 between 100 and 4 K is correlated with the inhomogeneities in fluorescence emission and energy transfer which develop in this temperature range. A simple model may be constructed where the antenna consists of clusters of about 60 BChls 880 separated by clusters of BChl 896 to explain the effects observed (Fig. 9). However, it is also possible that a spectral inhomogeneity within BChl 880 is involved. Evidence for such an inhomogeneity was recently obtained from the low-temperature absorption and flash-induced absorption difference spectra of *Rb. sphaeroides* and *R. rubrum* [15,21].

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