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Fluorescence quenching in *Heliobacterium chlorum* by reaction centers in the charge separated state

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By means of short laser flashes of varying intensity, followed by weak xenon flashes to monitor the fluorescence, the relation was studied between the fluorescence yield of bacteriochlorophyll (BChl) *g* and the redox state of the reaction center in membranes of *Heliobacterium chlorum*. At room temperature, as well as at 4 K, a decrease of the fluorescence yield was observed immediately after the laser flash. The fluorescence yield recovered with the same time constant as the re-reduction of the oxidized primary electron donor P-798$^+$, indicating that the fluorescence of antenna BChl in *H. chlorum* is quenched more strongly when the primary donor is oxidized than when it is reduced. At 4 K the fluorescence was reduced by 45% and at 300 K by 17% when P-798 was fully oxidized. At 4 K, the fluorescence quenching was stronger on the long-wavelength than on the short-wavelength side of the emission band. A linear relationship was observed between the fluorescence yield and the fraction of oxidized P-798$^+$, from which we conclude that, at least at 4 K, the photosystem of *H. chlorum* consists of separate photosynthetic units between which transfer of energy does not occur. The light-saturation curves for the fluorescence quenching were similar at room temperature and at 4 K, indicating approximately equal efficiencies for charge separation at the two temperatures. The photosynthetic system of *H. chlorum* is the first one where a stronger quenching by 'closed' than by 'open' reaction centers was observed. A possible mechanism for this quenching is discussed.

Introduction

Light absorbed by photosynthetic antenna pigments is transferred first as excitation energy within the antenna system and subsequently to the reaction centers, where charge separation is induced. The efficiency of these processes is normally very high and only a small percentage of the excitation energy is lost and partially observable as fluorescence. It has been known for many years that the yield of fluorescence may depend on the state of the reaction center. In purple bacteria the yield was found to be substantially lower when the reaction centers are in a photochemically active ('open') state, than when the reaction centers are in the inactive ('closed') state [1]. The effect was ascribed to different quenching efficiencies for the reduced and for the oxidized primary electron donor. In contrast to this, the primary electron donor of PS II, P-680, was reported to be an equally effective quencher of chlorophyll *a* fluorescence in the oxidized and in the reduced state [2,3] and the same may apply to PS I [4,5].

In this paper we report the results of measurements of the fluorescence yield of membranes of *H. chlorum*. This bacterium was discovered a few years ago and contains a new type of bacteriochlorophyll, BChl *g* [6]. The primary donor, P-798, is presumably a dimer of BChl *g* or of its 13^2-epimer [7]. Three spectral types of antenna BChl *g*, BChl *g* 778, BChl *g* 793 and BChl *g* 808 can be distinguished in the low temperature absorption spectrum [8]. Excitation energy is rapidly transferred between these BChl types [9] and at low temperature energy transfer appears to be unidirectional, BChl *g* 808 being the only species emitting fluorescence [10]. Recent evidence indicates that the antenna and reaction center are organized as a single

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Abbreviations: BChl, bacteriochlorophyll; P-798, primary electron donor; PS, photosystem.

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pigment protein complex [11], but otherwise little is known about the structure of the antenna and reaction center.

In this communication it will be shown that, in membranes of *H. chlorum*, inactive reaction centers, where P-798 is in the oxidized state, are more efficient traps for the excitation energy than photoactive reaction centers. The effect was observed both at 4 and at 300 K; at 4 K the fluorescence of BChl *g* is quenched by a factor of about 2 when P-798 is fully oxidized. At 300 K a quenching by a factor of 1.2 occurred. Analysis of the kinetics at low temperature indicates that the antenna of *H. chlorum* is organized in separate photosynthetic units, between which no transfer of energy occurs.

Materials and Methods

H. chlorum was grown in medium No. 1552 of the American Culture Collection, under a nitrogen atmosphere at 37°C and at a light intensity of 5000 lux from incandescent lamps. Membrane fragments were prepared as described by Van de Meent et al. [11]; they were suspended in a buffer containing 10 mM Tris (pH 8.0) or 10 mM cyclohexylaminopropane sulfonic acid (CAPS) (pH 10.4) and 10 mM sodium ascorbate. For each experiment the buffer was freshly prepared and purged with nitrogen. For experiments at low temperature a helium flow cryostate was used and 65% glycerol (v/v) was added to the samples in order to prevent crystallization. The absorbance of the samples at 532 nm was kept low (< 0.04 at 4 K; < 0.2 at 300 K) in order to achieve a homogeneous light distribution within the sample.

The apparatus used for the fluorescence measurements was described elsewhere [12]. The wavelength of the exciting laser pulse was 532 nm with a pulse length of approx. 25 ps. By means of a variable neutral density filter the energy could be adjusted between 0.05 and 5 mJ/cm^2. The relative yield of BChl *g* fluorescence was monitored by measuring the amount of fluorescence induced by a weak 10 μs xenon flash, given at a variable delay time after the laser flash. For each measurement a series of 15–100 laser flashes was used, given at a frequency of 0.5 Hz, unless otherwise indicated. The light of the xenon flash was filtered by means of a combination of filters to a band 40 nm wide, centered at 515 nm. The fluorescence passed a monochromator, set at a bandwidth of 10 and 3 nm at 300 and 4 K, respectively, for measurements of the kinetics; for fluorescence spectra the resolution was set at 1 nm. Both the excitation energy and the relative fluorescence yield were measured with integrating photodiodes (RCA-30810). Absorbance differences were measured as described by Smit et al. [13].

Results and Interpretation

Membranes of *H. chlorum* were cooled to 4 K and excited by a strong 25-ps laser flash to bring about charge separation. At a variable delay after the laser flash a weak xenon flash was used to monitor the fluorescence. Fig. 1 (△) shows the relative fluorescence due to the xenon flash as a function of the delay between the two flashes. The fluorescence was normalized to the fluorescence without preceding laser flash. The results show that the fluorescence was strongly quenched when measured immediately after the laser flash. The yield increased again with increasing delay time between the flashes and eventually reached approximately the original level. The mono-exponential time constant for recovery of the fluorescence was 2.3 ms, the same time constant as observed for re-reduction of P-798$^+$ by Smit et al. [14] by means of absorbance difference measurements. Smit et al. [14] also observed a small (6%) component which was irreversible on a ms time scale and which is also apparent in Fig. 1. From these results we conclude that the reaction center in the state P-798$^+$X$^-$, where X denotes a secondary electron acceptor, is an efficient quencher of BChl *g* fluorescence. Less quenching, but with the same kinetics, was observed when a 3-times weaker laser flash was used (○).

Fig. 2 shows the extent of fluorescence quenching as a function of the energy density of the laser flash. The results indicate that the fluorescence quenching is almost completely saturated (> 90%) at the highest energy density applied (approx. 3 mJ/cm^2). The saturation curve is similar to those obtained by Van Kan et al. [10] and by Kleinherenbrink et al. [15] by measuring

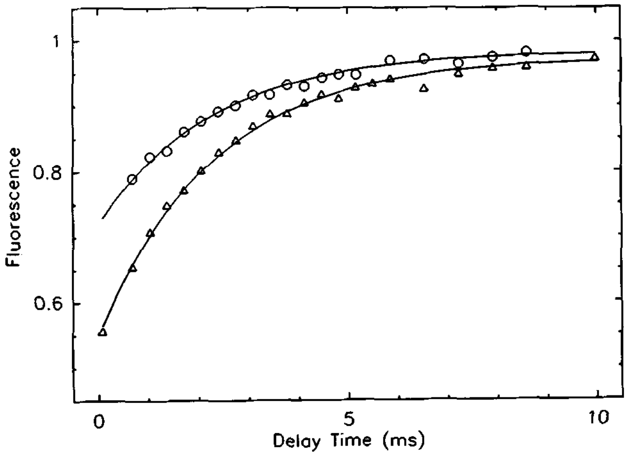

Fig. 1. Relative fluorescence at 825 nm of membranes of *H. chlorum*, measured at 4 K with a weak xenon flash at a variable time after a 25 ps laser flash. The fluorescence was normalized to the fluorescence without preceding laser flash. The energy density of the laser flash was approx. 1 mJ/cm^2 (circles) and 3 mJ/cm^2 (triangles), respectively. The lines represent the re-reduction of P-798$^+$ as measured by absorbance difference measurements [13].

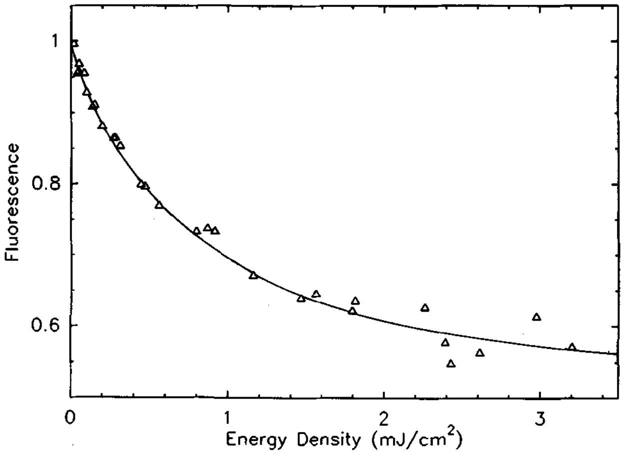

Fig. 2. Relative fluorescence at 4 K measured with the xenon flash 690 μs after a laser flash of variable energy density. The data were extrapolated to zero delay between the laser and the xenon flash.

the bleaching at 793 nm, due to formation of P-798$^+$, under the same conditions. This gives additional evidence that the fluorescence quenching is due to P-798$^+$. Assuming that P-798 is essentially 100% oxidized at 3 mJ/cm^2 we conclude that the fluorescence yield is quenched to 55% of the control level when P-798 is fully oxidized.

The observation that the recovery of fluorescence shows the same mono-exponential time constant as that of the absorbance indicates that the antenna of *H. chlorum* is organized in separate photosynthetic units. This can be analyzed quantitatively as shown in Fig. 3. The fluorescence yield observed from an antenna unit containing λ reaction centers, of which m centers are in the open state, is given by the following equation:

$$\phi(m) = \frac{k_f}{k_f + k_l + \frac{m}{\lambda}k_t^o + \left(1 - \frac{m}{\lambda}\right)k_t^c} \qquad (1)$$

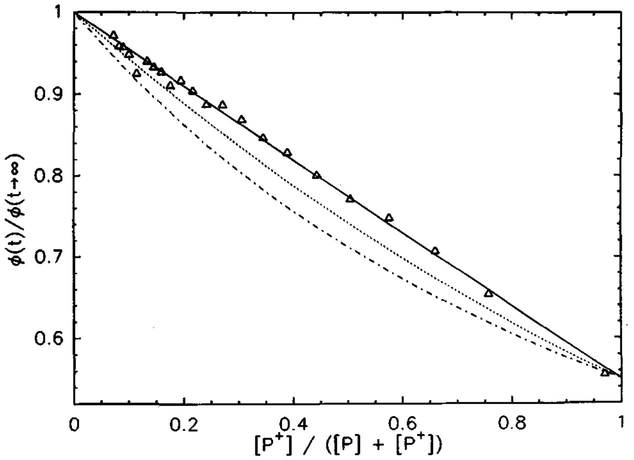

Fig. 3. Relative fluorescence as a function of the P-798$^+$ concentration, calculated according to eqn. (2) for $\lambda = 1$ (———), $\lambda = 2$ (······) and $\lambda = 20$ (·—·—·), respectively. The triangles were taken from the data in Fig. 1.

where k_t^o and k_t^c are the rate constants for trapping by reaction centers with P-798 and P-798$^+$, respectively, k_f is the rate constant for fluorescence and k_l accounts for all other loss processes. If we now assume that the number of open traps per antenna unit is given by a binomial distribution, the following relation between the fluorescence yield and the relative concentration $A(t)$ of P-798$^+$ is obtained:

$$\phi(A(t)) = \sum_{m=0}^{\lambda} \frac{k_f}{k_f + k_l + \frac{m}{\lambda}k_t^o + \left(1 - \frac{m}{\lambda}\right)k_t^c} \binom{\lambda}{m} (1 - A(t))^m \times A(t)^{\lambda - m} \qquad (2)$$

which is similar to the equation presented by Clayton [16]. From our data it follows that $(k_f + k_l + k_t^o)/(k_f + k_l + k_t^c) = 0.55$. In Fig. 3 we plotted $\phi(t)/\phi(t \to \infty)$ for various values of λ as a function of the relative P-798$^+$ concentration together with the experimental data taken from Fig. 1. It was assumed that $k_t^o/(k_f + k_l) = 10$. This value is not critical as long as it is much larger than 1, which is likely to be true for *H. chlorum*, since k_t^o is between 10 and 100 ns^{-1} [10] while $k_f + k_l$ is probably 1 ns^{-1} or less, as in purple bacteria [17]. The best fit is obtained with $\lambda = 1$ (a linear relationship); the lines for $\lambda = 2$ and $\lambda = 20$ show a deviation from the experimental points, especially in regions of strong quenching. This means that, at 4 K, each antenna unit of *H. chlorum* contains one reaction center and that no energy transfer between these units takes place.

To study the wavelength dependence of the fluorescence quenching we measured fluorescence spectra of *H. chlorum* membranes at 4 K. The spectra (Fig. 4) were measured with excitation by the xenon flash with or without a preceding saturating laser flash. Both spectra resemble the emission spectrum measured by Van Dorssen et al. [8], showing that essentially all emission comes from BChl *g* 808 at low temperature. However, the spectra of Fig. 4 not only differ in amplitude, as would be expected, but also in shape, as can be seen by dividing one spectrum by the other. This ratio (circles) is constant in the region above 825 nm, but at shorter wavelengths less quenching occurs and below 807 nm none at all.

The simplest way to analyze these results is by assuming that the spectrum without preceding laser flash is composed of two emission bands which are slightly shifted with respect to each other. If we assume that the fluorescence of the short-wavelength emitting pool of BChl *g* is not quenched by P-798$^+$, then the emission spectrum of the long-wavelength pool can be obtained by plotting the difference of the two emission spectra. This spectrum is shown in Fig. 4 by a broken line. The spectrum of the remaining component is not

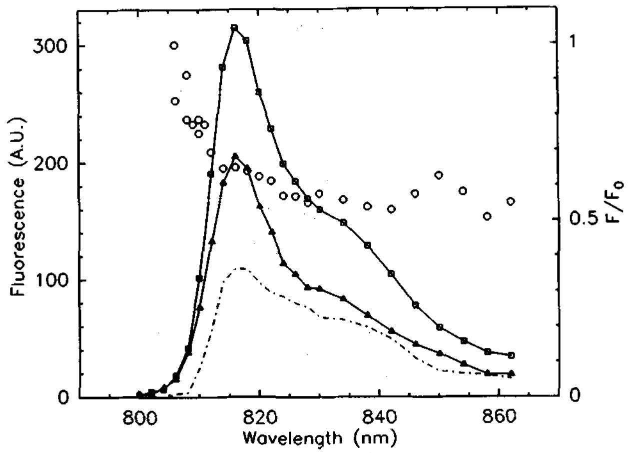

Fig. 4. Fluorescence intensity at 4 K measured directly after the laser flash (F, △) and without preceding laser flash (F_0, □) as a function of wavelength. The energy density of the laser flash was about 3 mJ/cm^2. The difference between the two spectra is given by the broken line; the ratio (F/F_0) by the circles.

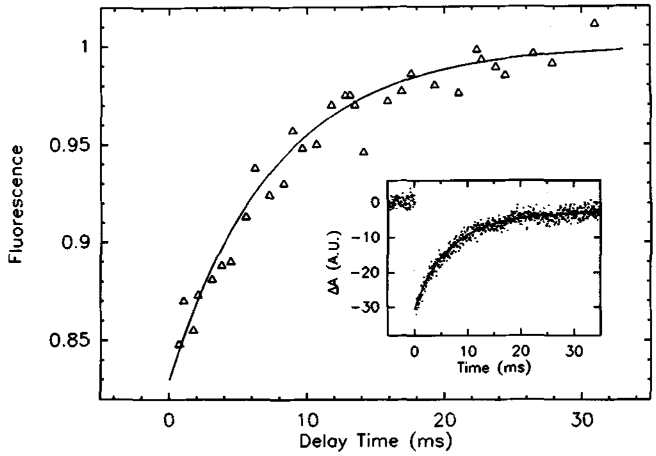

Fig. 5. Fluorescence recovery at 825 nm after a laser flash with an energy density of 3 mJ/cm^2 at 300 K at pH 8.0. The curve through the triangles represents a mono-exponential fit with a time constant ($1/e$) of 6.7 ms. The inset shows the kinetics of P-798$^+$ re-reduction as obtained by means of absorption difference (ΔA) measurements at 798 nm, fitted with two exponentials of 5.8 and 25 ms.

necessarily identical to that of the short-wavelength component, since the extent of quenching of the long-wavelength component by P-798$^+$ may be considerably less than 100% and therefore the contribution by the long-wavelength component to the fluorescence spectrum is not known. Nevertheless, it is clear that the peak positions are only slightly different for the two pools of BChl *g*; even if it is assumed that above 825 nm all fluorescence is due to the long-wavelength component, a difference of only 2 nm in peak positions (814 and 816 nm, respectively) is obtained (not shown).

Quenching experiments were also done at room temperature. However, these experiments were complicated by two factors: (1) the fluorescence yield is at least 20-times lower than at 4 K with a corresponding decrease in signal-to-noise ratio [8]; and (2) the yield of fluorescence was not stable for the duration of the experiment. Since at pH 8.0 the fluorescence yield was found to increase by a few percent during one flash series, we used a fresh sample for each measurement and performed most of the experiments at pH 10.4, where the stability was somewhat better. In continuous light large, slow changes of the fluorescence were observed, which precluded the use of continuous illumination to obtain a better signal-to-noise ratio.

The relative yield of fluorescence at 300 K as a function of the delay between the laser and the xenon flash at pH 8.0 is shown in Fig. 5. Like at 4 K, the fluorescence is quenched after a laser flash. A mono-exponential recovery of the fluorescence yield was observed with a time constant of 6.7 ± 0.5 ms. The same time (5.8 ± 0.5 ms) was observed in the re-reduction P-798$^+$ as measured by the kinetics of the absorbance at 798 nm after a flash (Fig. 5, inset). A second, slow phase (25 ms) was also present in the absorbance kinetics, which appeared to be absent in the kinetics of the fluorescence recovery. However, the amplitude of this slow phase was found to depend strongly on the intensity of the measuring beam. At the lowest intensity which could be usefully applied it amounted to about 15% of the total absorbance change, i.e., significantly less than earlier reported [13].

The same fluorescence kinetics were observed at pH 10.4 (Fig. 6). This figure also shows that the kinetics were only slightly affected by the presence of 70% glycerol, added to increase the viscosity of the medium. The fluorescence recovery was slowed down to about 8 ms and in addition a much slower component appeared. The flash frequency had to be reduced to about 0.1 Hz to allow complete re-reduction of P-798$^+$ between the flashes. A slowing down of the kinetics

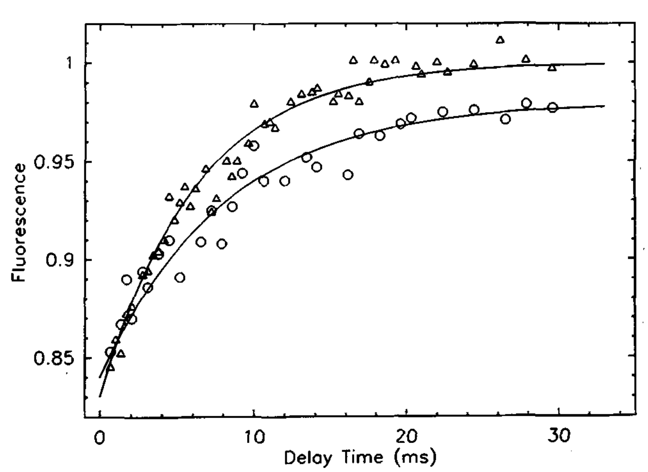

Fig. 6. Fluorescence recovery with (○) and without (△) 70% glycerol added, measured at pH 10.4. The data in the absence of glycerol were fitted with a mono-exponential decay time of 6.2 ms. With glycerol a time constant of 8.0 ms is obtained, together with an 'irreversible' component of 13%.

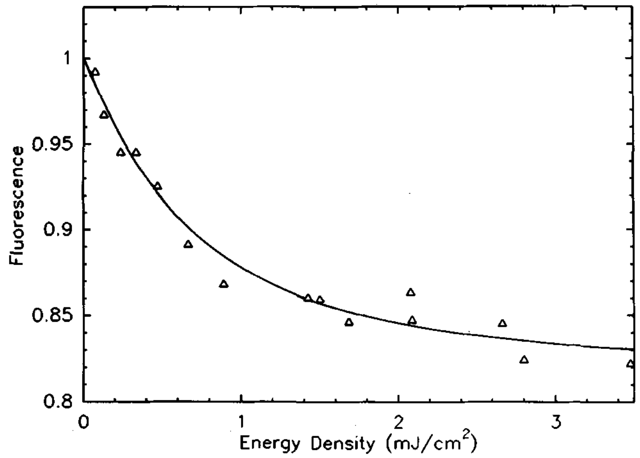

Fig. 7. Relative fluorescence at 300 K as a function of the energy density of the laser flash, measured at pH 10.4 with a time delay of 690 μs and extrapolated to $t = 0$.

was also observed in absorbance change measurements (not shown). These results give additional evidence that the fluorescence quenching is due to P-798$^+$ and indicate that its re-reduction is not a diffusion controlled reaction.

The intensity dependence of the quenching at 300 K is shown in Fig. 7. The saturation curve was similar to that obtained at 4 K, in agreement with measurements of flash induced absorbance changes [9,10]. This indicates that the effficiency of charge separation is about equal at both temperatures. From Fig. 7 we estimate that, at room temperature, the fluorescence yield at saturating intensity and $t = 0$ is 83% of the control.

Discussion

The most striking result reported here is the observation that 'closed' reaction centers of *H. chlorum* are more efficient quenchers for the excitation energy than 'open' reaction centers. The effect not only occurs at 4 K, but, to a lesser extent, also at room temperature. This indicates that the quenching is due to the oxidized primary electron donor P-798$^+$, rather than to a reduced electron acceptor with the same kinetics as P-798$^+$. At room temperature gramicidin did not affect the fluorescence quenching, indicating that the effect is not caused by a pH gradient or electrical potential over the membrane. Fluorescence emission spectra at 4 K indicate that the fluorescence quenching occurs in a long-wave form of BChl *g* 808.

A possible mechanism for the quenching might be energy transfer from BChl *g* 808 to P-798$^+$. The absorption band of P-798 is located at significantly shorter wavelength than that of BChl *g* 808. This means that the Förster overlap integral of BChl *g* 808 emission with P-798$^+$ absorption may be larger than with P-798 and thus a higher transfer rate to P-798$^+$ than to P-798 will occur. Preliminary experiments indicate that also in *Rhodopseudomonas viridis*, where the primary donor absorbs at significantly shorter wavelength than most of the antenna [18], a decrease of the fluorescence occurs at 4 K when the primary donor is oxidized. In other species of purple bacteria the overlap with the oxidized primary donor may be smaller, as compared to that of the reduced donor, thus leading to an increase of the fluorescence upon oxidation [1]. It should be mentioned that our results do not necessarily imply that BChl *g* 808 or its long-wave form is involved in energy transfer to 'open' reaction centers, as suggested by Smit et al. [14].

The high quenching efficiency by the 'closed' reaction centers may provide a useful mechanism to avoid photodamage by excess excitations in the antenna, which damage is especially likely to occur under aerobic conditions [19]. In this connection it should be mentioned that the mechanism which operates in purple bacteria, that is by the formation of carotenoid triplets by triplet-triplet energy transfer from BChl, is not present in heliobacteria [15].

We further observed that, since the saturation curves of Figs. 2 and 7 are almost identical, the efficiencies of charge separation at 300 and 4 K are about equal. At first sight this observation would seem to conflict with the strong increase in fluorescence yield upon cooling. However, it should be kept in mind that the fluorescence at room temperature is very weak, about 20-times lower than in *R. rubrum* and therefore a several fold increase in the fluorescence yield can still be reconciled with a fairly high yield of charge separation at low temperature.

Our results also show that at 4 K the photosynthetic units in *H. chlorum* are separated from each other. Unfortunately, at 300 K, the scatter in the fluorescence quenching measurements is too large and the quenching too small to draw conclusions about the antenna size. Singlet-singlet annihilation measurements to resolve this question so far gave inconclusive results. It may be noted that the presence of separate photosynthetic units (at room temperature) in heliobacteria would be advantageous to obtain a high efficiency of photochemistry. Whereas in purple bacteria connected units [12,21] provide a way to optimize the yield of photochemistry when part of the reaction centers are 'closed', in heliobacteria the 'closed' reaction centers would lower the yield of photochemistry by acting as sinks, draining away the excitation energy from neighboring photosynthetic units.

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References

1 Vredenberg, W.J. and Duysens, L.N.M. (1963) Nature 197, 355–357.

2 Butler, W.L. (1972) Proc. Natl. Acad. Sci. USA 69, 3420–3422.

3 Den Haan, G.A., Warden, J.T. and Duysens, L.N.M. (1973), Biochim. Biophys. Acta 325, 120–125.

4 Butler, W.L. (1978) Annu. Rev. Plant Physiol. 29, 345–378.

5 Klug, D.R., Giorgi, L.B., Crystall, B., Barber, J. and Porter, G. (1989) Photosynth. Res. 22, 277–284.

6 Gest, H. and Favinger, J.L. (1983) Arch. Microbiol. 136, 11–16.

7 Kobayashi, M., Van de Meent, E.J., Erkelens, C., Amesz, J., Ikegami, I. and Watanabe, T. (1991), Biochim. Biophys. Acta 1057, 89–96.

8 Van Dorssen, R.J., Vasmel, H. and Amesz, J. (1985) Biochim. Biophys. Acta 809, 199–203.

9 Nuijs, A.M., Van Dorssen, R.J. and Duysens, L.N.M. (1985) Proc. Natl. Acad. Sci. USA 82, 6865–6868.

10 Van Kan, P.J.M., Aartsma, T.J. and Amesz, J. (1989) Photosynth. Res. 22, 61–68.

11 Van de Meent, E.J., Kleinherenbrink, F.A.M. and Amesz, J. (1990) Biochim. Biophys. Acta 1015, 223–230.

12 Bakker, J.G.C., Van Grondelle, R. and Den Hollander, W.T.F. (1983) Biochim. Biophys. Acta 725, 508–518.

13 Smit, H.W.J., Amesz, J. and Van der Hoeven, M.F.R. (1987) Biochim. Biophys. Acta 893, 232–240.

14 Smit, H.W.J., Van Dorssen, R.J. and Amesz, J. (1989) Biochim. Biophys. Acta 973, 212–219.

15 Kleinherenbrink, F.A.M., Aartsma, T.J. and Amesz, J. (1991) Biochim. Biophys. Acta 1057, 346–352.

16 Clayton, R.K. (1967) J. Theor. Biol. 14, 173–186.

17 Sebban, P., Jolchine, G. and Moya, I. (1984) Photochem. Photobiol. 39, 247–253.

18 Holt, A.S. and Clayton, R.K. (1965) Photochem. Photobiol. 4, 829–831.

19 Krinsky, N.I. (1979) Pure Appl. Chem. 51, 649–660.

20 Deinum, G., Aartsma, T.J., Van Grondelle, R. and Amesz, J. (1989) Biochim. Biophys. Acta 976, 63–69.