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Linear dichroism and fluorescence emission of antenna complexes during photosynthetic unit assembly in *Rhodopseudomonas sphaeroides*

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Photosynthetic membrane formation was induced in a nonpigmented, aerobic culture of *Rhodopseudomonas sphaeroides*. During the course of the experiment, the bacteriochlorophyll content of the cells increased approx. 40-fold [1]. Membranes prepared from pigmenting cells were analysed by fluorescence emission and excitation spectroscopy at 4 K; at all stages efficient energy transfer was observed between B850 and B875 bacteriochlorophylls within the light-harvesting system [2]. A light-harvesting domain containing several interconnected traps (reaction centres) and about 200 B875 bacteriochlorophylls was present in membranes from cells grown under high aeration. Over the course of the experiment the size of this domain increases 5-fold in intracytoplasmic membranes, with the result that the total light-harvesting network is composed of 3000 bacteriochlorophyll molecules; these surround and interconnect approx. 30 reaction centres. We estimate the average reaction centre–reaction centre distance in mature intracystoplasmic membranes to be about 15 nm and the average bacteriochlorophyll–bacteriochlorophyll distance to be about 1.5 nm [3]. An examination of the linear dichroism properties of stretched pigmenting membranes demonstrated that all orientations of pigments are maintained during development, the orientation of the B875 absorbance band being strongly parallel to the stretch axis. The expansion of the photosynthetic unit during development appears to involve the addition of B800–850 antenna to form increasingly random aggregates around highly ordered B875 antenna complexes.

Introduction

Rhodopseudomonas sphaeroides is a facultatively photosynthetic bacterium which contains an intracytoplasmic membrane system that houses the photosynthetic apparatus. This apparatus is composed of photochemical reaction centres surrounded and interconnected by two different types of light harvesting (antenna) complexes designated B875 and B800–850 according to the near infra-red absorption of the bacteriochlorophyll molecules in the complex. *Rps. sphaeroides* synthesises appreciable quantities of intracytoplasmic membrane during chemoheterotrophic growth in the dark in the presence of low (at most 2.5%) oxygen [1,2]; at higher concentrations of oxygen, the formation of intracytoplasmic membranes is repressed. The

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Abbreviations: B800, B850 and B875, bacteriochlorophyll molecules having absorption maxima at 800, 850 and 875 nm, respectively; BChl, bacteriochlorophyll; LD, linear dichroism.

transition of a non-pigmented cell to the fully pigmented state under the control of oxygen tension therefore presents an extremely useful model system for the study of membrane biogenesis.

Intracytoplasmic membranes arise as invaginations of a cytoplasmic membrane [3]. Fractionation experiments by Niederman et al. [4] demonstrated that small and large invaginations of the cytoplasmic membrane are separable by sucrose density gradient centrifugation giving rise in the gradient to an upper pigmented band and an intracytoplasmic membrane band composed of a nearly uniform population of closed vesicles. Pulse labelling studies [4,5] have demonstrated that during development of the intracytoplasmic membrane the polypeptide components of the pigment-protein complexes are inserted preferentially into the upper pigmented membrane. A study of electron transport kinetics [6] demonstrated that upper pigmented membranes do not exhibit the light-driven reactions characteristic of mature intracytoplasmic membrane, and that the upper pigmented membrane has a relatively low content of B800–850 light harvesting complex. It is the purpose of this work to examine the light-harvesting function of developing upper pigmented and intracytoplasmic membranes by the application of fluorescence and linear dichroism techniques. The efficiency of energy transfer within the developing photosynthetic apparatus has been measured using low-temperature fluorescence spectroscopy. We have also applied a technique involving measurement of the quenching of singlet-singlet bacteriochlorophyll fluorescence yield. This enabled Van Grondelle and co-workers [7] to quantify the number of bacteriochlorophyll molecules that form a functional light-harvesting domain in detergent solubilized antenna complexes from some photosynthetic bacteria. Here we quantify the sizes of domains in upper pigmented and intracytoplasmic membranes as a function of development. Such studies complement earlier fluorescence work on the emission properties of the developing photosynthetic apparatus in whole cells [8] and crude membrane extracts [9]. Hunter and co-workers [10] examined the fluorescence of purified upper pigmented and intracytoplasmic fractions from photosynthetically grown cells and observed anomalously high emission from the B850 bacteriochlorophylls in the upper pigmented membrane. In this paper we report the results of detailed measurements on purified membranes from a dark, chemoheterotrophically grown culture in which the oxygen content of the medium is sufficiently low to derepress pigment synthesis. The results demonstrate that energy transfer between B850 and B875 bacteriochlorophyll molecules is highly efficient in both upper pigmented and intracytoplasmic membranes, and that the orientations of the pigments with respect to the plane of the membrane are maintained throughout photosynthetic unit assembly. A preliminary report of these studies has appeared elsewhere [11].

Materials and Methods

Induction of pigment synthesis

Rps. sphaeroides NCIB 8253 was grown in the dark under conditions of high aeration at 30°C in 1 l baffled conical flasks containing 400 ml Cohen-Bazire medium [12]. The shaker speed was 180 rpm. When the absorbance at 680 nm (A_{680}) was approx. 1.0 the cells were harvested and either used for preparation of 'time zero' membranes (see below) or for the induction of pigment synthesis. In the latter case the cells were resuspended in fresh medium ($A_{680} = 2.2$; A_{850}: $A_{680} = 0.65$; A_{850}: $A_{875} = 1.06$) and 900 ml of cell suspension dispensed into 1 l flasks (no baffles). After 5 or 21 h growth at 30°C in the dark with a shaker speed of 180 rpm, the pigmented cells were harvested by centrifugation.

Isolation of membrane fractions

This followed the procedure outlined previously [10]. Membrane fractions from several sucrose density gradients were pooled and concentrated in an Amicon Ultrafiltration cell and mixed with glycerol to a final concentration of 50% (v/v) for storage at −20°C.

Low temperature emission, excitation, absorption and linear dichroism (LD) spectra were obtained using a single-beam spectrophotometer described elsewhere [13]. The fluorescence yield as a function of the energy of a 30 ps, 532 nm laser flash was measured as described in Ref. 7. To keep the samples clear upon cooling, the membranes in glycerol were mixed with 50% (v/v) glycerol/0.5

M sucrose. For LD spectra the membranes were oriented using a polyacrylamide gel pressing technique [14]. Bacteriochlorophyll was determined by acetone/methanol (7 : 2, v/v) extraction using the extinction coefficient given by Clayton [15].

Results and Discussion

Absorbance and fluorescence emission spectra

The duration of induction of pigment synthesis was approx. 21 h, and in this time the amount of bacteriochlorophyll per cell increased 40-fold. Three time points, 0, 5 and 21 h were chosen to describe the induction sequence. Absorbance and fluorescence emission spectra for upper pigmented and intracytoplasmic membranes are shown in Fig. 1. These spectra show the qualitative differences between the various membrane fractions through the time-course of induction, but show no quantitative information on the amounts of upper pigmented and intracytoplasmic membranes present or on the fluorescence yield. In agreement with earlier observations [4,5] the upper band was the major membrane fraction at time zero. As a result of further pigment synthesis and invagination the membranes assume the isolation characteristics of intracytoplasmic membrane vesicles in sucrose density gradients and become the major pigmented fraction.

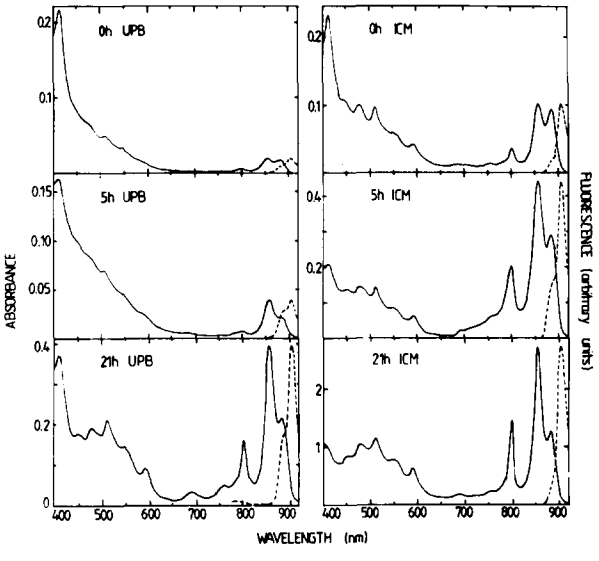

Fig. 1. Absorbance (————) and emission (------) spectra of membranes at 4 K; excitation at 590 nm. The spectra were normalised at their near infrared maxima. The pigment levels at 0, 5 and 21 h, were 5, 16 and $210 \cdot 10^4$ BChl molecules per cell, respectively. UPB, membranes recovered from the upper pigmented band of the sucrose density gradient (see Materials and Methods); ICM, intracytoplasmic membranes recovered from the lower band of the gradient.

The first event in photosynthetic membrane assembly is the insertion of pigment proteins (mainly B875 and reaction centres) into the cytoplasmic membrane, and therefore the zero-time upper band has a small absorbance at 850 nm in relation to the cytochrome absorbing maximally at 420 nm. In this respect, upper pigmented and intracytoplasmic membrane fractions differ at all stages of development, in agreement with earlier work [6]. Measurement of absorbance and fluorescence emission at 4 K allows differences in B850 and B875 content to be examined. 'Early' membranes contain a relatively high amount of B875 but B800–850 is the major antenna species at 5 h and 21 h. The major fluorescence emission peak is around 900 nm for all membranes, due to energy transfer from the network of B800–850 antenna to B875. In cases where this energy transfer is impaired, some emission would be expected from B850 at 865 nm and for each upper pigmented sample we did observe more fluorescence at 865 nm than for the appropriate intracytoplasmic membrane sample. Since earlier work on membranes from photosynthetically grown cells [9] had shown similar fluorescence behaviour at room temperature but no calculation of the energy-transfer efficiency had been made, we attempted to quantify the efficiency of energy transfer.

A series of emission and excitation spectra was obtained for each membrane sample (not shown) and used to calculate the percentage efficiency of energy transfer within the expanding photosynthetic unit (Table I). The data clearly shows that from the earliest stage of pigmentation, energy transfer from B850 to B875 bacteriochlorophylls is highly efficient. Transfer of excitation energy from carotenoid to bacteriochlorophyll (510 → 590 nm; 550 → 590 nm) is also efficient in the intracytoplasmic fraction at time zero, although this parameter does increase slightly over the 21 h induction process.

TABLE I

THE EFFICIENCY OF ENERGY TRANSFER WITHIN THE EXPANDING PHOTOSYNTHETIC UNIT OF UPPER PIGMENTED AND INTRACYTOPLASMIC MEMBRANES

*, Measurements were unreliable due to light scattering artefacts at these wavelengths.

Membrane preparation		Percentage transfer efficiency			
		510 → 590 nm	550 → 590 nm	800 → 850 nm	850 → 875 nm
Upper pigmented membranes	0 h	*	*	75	94
	5 h	*	*	74	94
	21 h	88	80	82	98
Intra-cytoplasmic membranes	0 h	80	90	89	98
	5 h	82	84	86	100
	21 h	100	100	100	95

Singlet–singlet quenching of the bacteriochlorophyll fluorescence yield

In order to gain an idea of the number of bacteriochlorophyll molecules linked to form an efficient light-harvesting network, a so-called 'domain', singlet–singlet quenching of the bacteriochlorophyll fluorescence yield was measured as a function of the excitation intensity (Fig. 2). This method has been used to estimate the size of antenna complexes isolated from various photosynthetic bacteria [7] and to calculate domain sizes in intracytoplasmic membrane vesicles (chromatophores) of *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata* [16,17]. In this work with *Rps. sphaeroides* fluorescence yield was measured at 900 nm as a function of laser pulse intensity, and it was found that the results obtained for 21 h intracytoplasmic membranes were strongly analogous to those obtained for chromatophores of *Rps. capsulata* [17], taking into account the difference in absorption cross-section at the excitation wavelength (532 nm) between the two species.

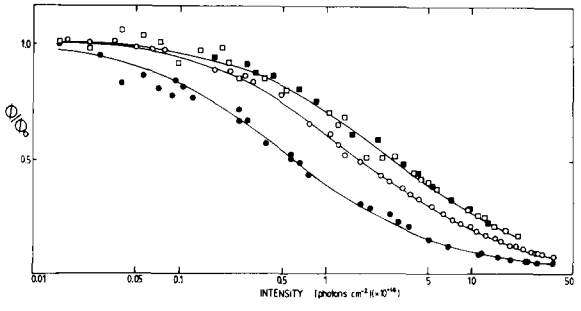

Fig. 2. Fluorescence yield as a function of flash intensity for membranes isolated from *Rps. sphaeroides*. The fluorescence of all preparations was detected at 900 nm. The absorbance for all the samples at 532 nm was 0.1 ϕ/ϕ_0 was normalised to 1.0 at low intensities. The symbols for the curves are: □, 0 h upper pigmented membranes; ■, 0 h intracytoplasmic membranes; ○, 21 h upper pigmented membranes; ●, 21 h intracytoplasmic membranes.

For 0 h intracytoplasmic and upper pigmented membranes identical fluorescence quenching profiles were observed, but compared to mature (21 h) intracytoplasmic membrane the experimental points that correspond to the same degree of fluorescence quenching occurred at about 5-fold higher intensities. For 21 h upper pigmented membranes, the fluorescence quenching was intermediate between 21 h intracytoplasmic membrane and 0 h intracytoplasmic and upper pigmented membranes.

All these results can be fitted reasonably well with an expression given by Swenberg et al [18]:

$$\phi = \frac{\phi_0}{aI} \ln (1 + aI) \qquad (1)$$

where I is the intensity measured in photons per cm^2, ϕ_0 is the low-intensity fluorescence yield and a is a fit parameter that defines the position of the curve relative to the intensity axis. For 21 h intracytoplasmic membranes $a = 4.7 \cdot 10^{-14}$ cm^2 per photon and for both 0 h membranes $a = 9.4 \cdot 10^{-15}$ cm^2 per photon. The fits are shown in Fig. 2.

We have also used a more general expression

given by Paillotin et al. [19] in which the fluorescence yield vs. pulse intensity curve is described by two parameters: Z, the number of excitations generated per domain, and $r = 2\gamma_1/\gamma_2$, where γ_1 is the rate of monoexcitation decay and γ_2 the rate of excitatation annihilation per pair of excitations [16,19]. For 21 h intracytoplasmic and upper pigmented membranes we calculate $r > 3$ from the experimental data. For both zero time membrane samples $r = 2$–3. We note that according to Paillotin et al. [19] the shape of the fluorescence quenching curve becomes indistinguishable from that given by Eqn. 1 for large values of r. This is the reason why Eqn. 1 gives a good fit to the experimental data for 21 h membranes. For zero time membranes the experimental data in the low-intensity region scatter above the curve predicted by Eqn. 1 and this results in a somewhat smaller value of r.

Thus either a change in Z or a change in r (or in both) may effect the pulse-intensity dependence of the fluorescence yield. Smaller values of r will shift the curve to lower intensities and below $r \simeq 3$ will change its shape; smaller values of Z will shift the curve to higher intensities.

A change in Z may occur due to a different efefctive absorption cross-section per B875 molecule. However, the excitation spectra of the B875 emission were roughly equal for all preparations, indicating that in each case about equal numbers of photons were absorbed per bacteriochlorophyll *a* molecule at the same pulse intensity. Because the B850/B875 ratio approximately doubles over 21 h and assuming over 90% efficient energy transfer to B875, a 33% increase in the total cross-section per B875 may be expected in the 21 h preparations. This may explain a small fraction of the shift to lower intensity of the fluorescence quenching curve obtained for 21 h intracytoplasmic membranes.

A change in r may occur due to a change in γ_1 or γ_2. The low-intensity fluorescence yield of 21 h intracytoplasmic membranes is about 2-fold lower than in zero hour intracytoplasmic membranes implying an increase in γ_1. This may easily compensate for the effect mentioned above on the increased effective absorption cross-section of the fluorescence quenching curve of 21 h intracytoplasmic membranes.

Thus we are left with a change in domain size to explain the difference between zero hour upper pigmented and intracytoplasmic membranes, and 21 intracytoplasmic membranes. A largely decreased domain for zero hour membranes will on one hand lead to a decreased value or r (via an increase in γ_2) and a decreased value of Z. Assuming $r \simeq 2$ for zero time membranes and $r \simeq 5$ for mature 21 h intracytoplasmic membrane and a domain size of 100–200 B875 molecules for the former and about 1000 B875 molecules for the latter, we arrive at a satisfactory fit of the fluorescence quenching curves. For 21 hour upper pigmented membranes a domain size of about 300 B875 molecules can be estimated with $r \simeq 3$. Part of this apparent increase in domain size in the upper pigmented membrane could be attributable to increasing contamination with intracytoplasmic membranes. However, the method used to separate the two membrane fractions – sucrose density gradient centrifugation – has been shown in past work on photosynthetically grown cells to produce upper pigmented and intracytoplasmic membranes of widely differing radiolabelling characteristics (for example, see Refs. 4 and 5). This would not be possible if contamination of upper pigmented membranes with intracytoplasmic membranes was a serious problem.

In conclusion, even in the early stages of photosynthetic unit assembly, domains are present that contain at least 100–200 connected B875 molecules (or 3–6 connected reaction centres). These domains may increase 5-fold in size over the 21 h of this experiment. Because cell bacteriochlorophyll content increases 40-fold overall, the membrane invagination that occurs is consistent with the fact that membrane area must increase to accommodate more domains.

Linear dichroism

The linear dichroism properties of the various membrane fractions were investigated in order to provide information on the orientation of the antenna complexes during photosynthetic membrane assembly. In order to provide additional information on the use of the polyacrylamide gel squeezing method for bacterial photosynthetic membranes, upper pigmented and intracytoplasmic membrane fractions from cells grown at high or low light intensity were included in this analysis

for comparison. Membranes from cells grown at low light intensity contain a relatively high proportion of B800–850 antenna with respect to B875 [20].

Fig. 3 shows the linear dichroism of membrane preparations versus wavelength. The Q_y transitions of B850 and B875 bacteriochlorophylls are oriented parallel to the stretch axis, and further analysis of linear dichroism/absorbance (LD/A) values (Table II) reveals that the Q_y transition of B875 is particularly strongly oriented parallel to the stretch axis, and therefore the plane of the membrane surface. This is observed in all membranes at all stages of development. One general effect of membrane development during low oxygen growth or low light phototrophic growth is the increased size of the B800–850 antenna 'lake', more specifically in the intracytoplasmic membrane [5]. The LD/A values for B850 in the intracytoplasmic fraction are lower than those for the upper fraction in all growth conditions, the difference being least at the start of pigment synthesis in low-oxygen conditions. This suggests that the assembly of a large B800–850 array in the intracytoplasmic membrane results in some degree of disorder in the orientation of the Q_y transitions of the bacteriochlorophyll molecules. In contrast, the B875 complement of the cell maintains a highly ordered state; in all membrane samples the LD/A value for B875 is significantly higher than for B850. This may reflect some intrinsic property of B875 antenna, or just the fact that B875 aggregates never achieve the same size as B800–850 aggregates.

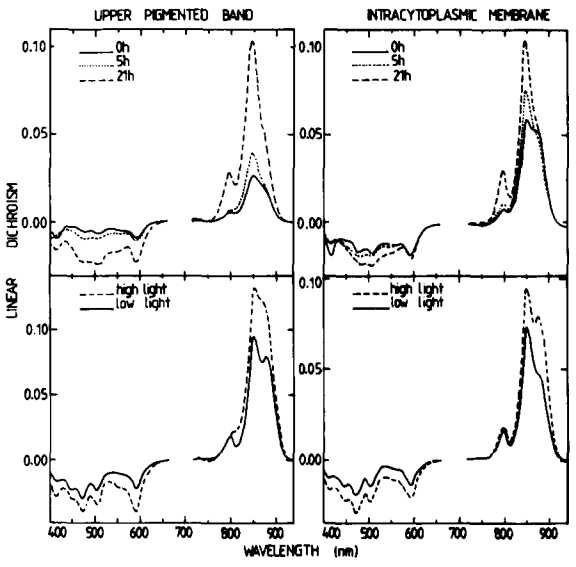

Fig. 3. Linear dichroism ($A_{\parallel} - A_{\perp}$) of membranes isolated from pigmenting *Rps. sphaeroides*. The spectra were recorded at room temperature. High and low light refer to 500 ml anaerobic cultures, irradiated by tungsten filament light. The power outputs of the light sources were 1760 and 66 $W \cdot m^{-2}$, respectively, at a distance of 20 cm. The pigment levels of the high and low light cultures were 1.4 and $4 \cdot 10^6$ BChl molecules cell, respectively.

TABLE II

LD/A RATIOS FOR UPPER PIGMENTED AND INTRACYTOPLASMIC MEMBRANES ISOLATED FROM PIGMENTING CELLS

The LD/*A* ratios $(A_{\parallel} - A_{\perp})/A$ for these samples were calculated from the spectra in Fig. 3 and from a series of absorbance spectra in acrylamide recorded on the same apparatus used for LD measurements.

Membrane preparation		Linear dichroism/absorbance		
		800 nm	850 nm	875 nm
Upper pigmented membrane	0 h low O_2	0.18	0.31	0.37
	5 h low O_2	0.25	0.39	0.43
	21 h low O_2	0.30	0.40	0.45
	high light photosynthetic	0.24	0.47	0.53
	low light photosynthetic	0.18	0.30	0.59
Intra-cytoplasmic membrane	0 h low O_2	0.14	0.28	0.41
	5 h low O_2	0.20	0.34	0.51
	21 low O_2	0.18	0.25	0.41
	high light photosynthetic	0.10	0.26	0.40
	low light photosynthetic	0.13	0.20	0.35

Concluding remarks

The data presented here demonstrated that light is harvested extremely efficiently even at the earliest stage of photosynthetic development studied here, namely the upper pigmented membrane isolated from cells grown under conditions of aeration sufficient to repress almost all bacteriochlorophyll synthesis. This membrane possesses a small and well ordered aggregate of B875 antenna which interconnects and surrounds 3–6 reaction centres. We conclude from the measurements of domain size in Fig. 2 that although some limited expansion of the light-harvesting network is possible in the upper pigmented membrane, the majority of bacteriochlorophyll accumulation occurs in the intracytoplasmic membrane largely by the addition of B800–850 antenna [21].

In the earliest stages of pigmentation there are of the order of 50 BChl per reaction centre with 30 BChl per reaction centre as the lower limit [9]. This number may approach 150 BChl per reaction centre in cells grown photosynthetically (data not shown). It is clear that the possibility of increasing this parameter up to 3-fold is insufficient to account for the overall increase in bacteriochlorophyll content from approximately $5 \cdot 10^4$ to $2.1 \cdot 10^6$ molecules per cell. The main devices for increasing the bacteriochlorophyll content of the cell are therefore (a) an increase in the amount of bacteriochlorophyll per membrane surface area, and (b) the expansion of the membrane surface area within the cell. In the discussion of their fluorescence study [9]. Pradel and co-workers used a quantitative approach to show that with the available membrane surface area and the relatively small number of bacteriochlorophyll molecules in high aeration cells, random insertion of this pigment would not account for the high efficiency of 'interunit' energy transfer observed. This approach has been extended and the results are presented in Table III. Several more parameters can be estimated as a result of this analysis.

(i) Within an intracytoplasmic membrane invagination (or vesicle) containing up to 30 reaction centres, the average distance between reaction centres would be approx. 16 nm. Miller [22] has analysed membranes from *Rps. viridis* and measured a distance of 11.5 nm between reaction centres. The number for the average distance calculated here is therefore in reasonable agreement, taking into account that the 'lake' model [23] for the arrangement of the photosynthetic apparatus proposes that some reaction centres are relatively close together, interconnected by B875 antenna and that these reaction centre-B875 regions border large lakes of B800–850 antenna. The distance between reaction centres on opposite sides of a 'lake' would be more than 11.5 nm.

(ii) The requirements imposed on antenna BChl–BChl distance by energy transfer considerations limit the size of this parameter to approx. 2 nm [24,25]. The average BChl–BChl distance in a

TABLE III

CALCULATIONS ON THE NUMBER AND SIZE OF INVAGINATIONS IN PIGMENTING *RPS. SPHAEROIDES* MEMBRANES

Parameter	High aeration cells	Low aeration cells
Bacteriochlorophyll molecules per cell	$5 \cdot 10^4$	$2.1 \cdot 10^6$
Unit size BChl: reaction centre [a]	50	100
Reaction centres per cell	1000	$2 \cdot 10^4$
Number of BChl molecules connected for energy transfer	200 B875 BChl 7 reaction centres 300 BChl total	1000 B875 BChl 30 reaction centres 3000 BChl total
Number of invaginations per cell [b]	140	640
Membrane surface area	$4 \cdot 10^6$ nm^2	10^7 nm^2 [c]
Average distance between invaginations	170 nm	80 nm

[a] Calculated from absorbance spectra of membranes using the approximations of Crounse et al. [26] to estimate B875 absorbance, the extinction coefficient ($E_{mm} = 130$) in Ref. 20 to estimate B875 BChl, and the assumption that B875 BChl: reaction centre is constant around 30 [9,20,27].

[b] Calculated from the ratio BChl molecules per cell: number of connected BChl molecules, assuming one domain of energy migration per invagination. This number would, include upper pigmented and intracytoplasmic membrane invaginations, with the upper pigmented membrane forming an increasingly low proportion of the total as cell pigmentation increases.

[c] Calculated from the surface area of a rod-shape cell 2 μM long, 1 μM wide [9]; for low aeration cells an extra area equivalent to 640 chromatophore vesicles 50 nm in diameter [28] is added.

membrane vesicle 50 nm in diameter containing 3000 BChl molecules is 1.5 nm (100 BChl per reaction center; 30 reaction centers).

These estimates are consistent with the proposal that photosynthetic membrane development in this bacterium eventually results in the assembly of energy transfer domains each of which is equivalent to one intracytoplasmic membrane invagination – a 'chromatophore'.

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