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FORMATION AND DEVELOPMENT OF PHOTOSYNTHETIC UNITS IN REPIGMENTING *RHODOPSEUDOMONAS SPHAEROIDES* WILD TYPE AND "PHOFIL" MUTANT STRAIN

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Summary

The formation of the photosynthetic apparatus in the wild type *Rhodopseudomonas sphaeroides* and in the "Phofil" mutant was investigated by analyzing absorption and fluorescence parameters and the kinetics of fluorescence induction. Repigmentation was induced by transfer of bleached cells to semi-aerobic culture conditions.

1. In the "Phofil" mutant, functional photosynthetic units appear at pigment cellular contents which depend on the physiological state of the inoculum. The unadapted mutant can reach the functional units stage at a cellular pigment level 20 times lower than that of the wild type, although the size and composition of the units are identical in both strains. This rules out the hypothesis of photosynthetic units forming by random integration of pigments into the membrane. Moreover, the fact that units are separate at this stage (as shown by the exponential shape of fluorescence induction kinetics) suggests that the units' formation proceeds from discrete predetermined membrane sites.

2. In repigmenting wild type cells, the multistep assembly of bacteriochlorophyll complexes appears correlated to their organization in photosynthetic units as follows:

(i) During a first stage, *B*-875 is mostly synthesized, while the efficiency of transfer increases, suggesting that the pigments are initially interpersed at comparatively large average distances from each other in the bleached membrane.

(ii) After 1.5 h of repigmentation, the transfer and trapping efficiencies reach a maximum. The units (26 *B*-875 + 11 *B*-850 per center) are still separate, as shown by exponential fluorescence kinetics.

(iii) The increase in the units' size then essentially proceeds through *B*-850

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incorporation. Energy transfer between units occurs at a comparatively late stage, i.e., 5 h repigmentation.

Introduction

The size of the photosynthetic units in *Rhodopseudomonas sphaeroides* varies noticeably according to culture conditions. Aagaard and Sistrom [1] suggested that these variations are due to changes in the relative amount of light harvesting complex *B*-850, the other complex, *B*-875, remaining in a fixed proportion to the reaction centers (*P*-870). During the first steps of repigmentation of previously bleached cells, *P*-870 and *B*-875 are preferentially synthesized [2,3], yielding *B*-875-enriched units with a small size. The antenna increases later by addition of *B*-850.

The mechanism of sequential integration of the elements of the photosynthetic apparatus in facultative phototrophic bactera into the membrane is now generally accepted [3,5]. However, little data bear on the development of photosynthetic activity in repigmenting cells in order to correlate the multistep mechanism of photopigments' assembly and their organization into functional photosynthetic units.

Clayton [6] and recently, Monger and Parson [7] showed that, in pigmented cells, photosynthetic units are connected to each other, i.e., a common antenna is shared by several centers. However, there is no information on the formation of this connected state. One may think that the progressive addition of *B*-850 links previously unconnected units of *B*-875; or the connection is caused by the addition of new units; another possibility is that units are connected from their formation on, with a subsequent addition of the *B*-850 complexes on the periphery of the connected units zones.

In this paper, the formation of the photosynthetic units and their organization in the membrane have been investigated at various stages during repigmentation of aerobically grown *Rps. sphaeroides*. In addition to the parameters monitored in a similar experiment by Cellarius and Peters [8], we analyzed the kinetics of fluorescence induction giving information on the size of the units and on the degree of connection.

Peters and Cellarius [9] discussed the problem of whether pigment was incorporated (randomly) into the entire cytoplasmic membrane, or into discrete sites. They could not conclude on this matter for, in the wild type of *Rps. sphaeroides*, the appearance of functional units occurs at a stage where the cellular pigment content is rather high, so that a random coverage of the cytoplasmic membrane is not clearly inconsistent with the requirement of small bacteriochlorophyll distances for efficient energy transfer to occur. We report here similar experiments with the "Phofil" mutant of *Rps. sphaeroides* [10] which presents an anomalous adaption phase from aerobic to photosynthetic (or semi-aerobic) growth. According to their physiological state, the mutant cells integrate only a fraction ("unadapted" cells) or the whole ("adapted" cells) of synthesized pigments [11]. Depigmented cultures can be obtained which have different potentialities of membrane differentiation. This allows a study of the photopigments assembly at low level of pigment cellular content

(although the functional units stage has been reached), which favours the hypothesis of discrete sites for membrane pigmentation.

Materials and Methods

Organisms and growth conditions

Wild type *Rps. sphaeroides* (strain Y) and its descendent, the mutant "Phofil", were grown in a synthetic medium with succinate as carbon source [12]. Bleached cells were obtained by aerobic growth during 8 generations in the light in volumes of 20—30 ml. The bacteria were transferred in glass bottles (2 l), diluted with fresh medium up to a density of 10^8 cells per ml and pigment synthesis was induced by gassing with a 5% O_2/95% N_2 mixture.

Isolation of membrane extracts

All isolation procedures were performed at 0—5°C with $5 \cdot 10^{-2}$ M phosphate buffer, pH 7.5. Washed cells were resuspended to a concentration of 0.2 g wet weight per ml of buffer with DNAase (10 μg/ml), and passed twice through a French pressure cell (20 000 psi). The suspension was centrifuged twice at 10 000 $\times g$ for 10 min. The supernatant was centrifuged at 150 000 $\times g$ for 90 min. The pellet was resuspended in 10^{-2} M Tricine buffer/0.25 M sucrose, pH 7.4 and stored overnight at 0°C under Argon. Before use, membrane extracts were diluted to a concentration of bacteriochlorophyll of approx. 5 nmol per ml.

Bacteriochlorophyll determination

Bacteriochlorophyll was determined by acetone/methanol (7 : 2, v/v) extraction using the molar extinction coefficient given by Clayton [13]. Levels of light harvesting bacteriochlorophyll complexes in membrane extracts were determined from near infrared absorption spectra. Absorbance of the *B*-850 and *B*-875 bands were corrected by the method of Crounse et al. [14]. Extinction coefficients calculated by Sistrom [15] have been used. The amount of reaction center bacteriochlorophyll was determined from light-induced absorption changes at 870 nm on a Cary 14 R spectrometer used successively in the IR 1 and IR 2 modes. The amount of reaction center bacteriochlorophyll was calculated assuming and extinction coefficient of $1.16 \cdot 10^5 \cdot M^{-1} \cdot cm^{-1}$ [16].

Absorption spectra

Absorbance measurements were recorded on a Cary 14 R spectrometer. Levels of fluorescence intensity emitted by whole cells were detemined as previously [10].

Fluorescence induction measurements

1. Principle of method. It is well known that (bacterio)chlorophyll in functional photosynthetic membrane has a short fluorescence lifetime (and a low fluorescence yield) compared to that of the pigment in solution. This low fluorescence is primarily due to the efficiency of transfers within light-harvesting antenna which convey the electronic excitation to a reaction center

where it is trapped. This trapping turns the center into a high fluorescence form as long as the primary acceptor remains reduced. When a continuous illumination is set on, a rise of the fluorescence yield is observed. Analyzing the information from these fluorescence induction curves is made simpler when the reoxidation of the primary acceptors is blocked by an inhibitor such as *o*-phenanthroline [17,18]. Several parameters are then of interest.

(a) The rate of the induction kinetics (or the half-rise time) at a given intensity. It depends both on the rate of transfers within the antenna and on the size of the photosynthetic unit (number of harvesting pigments per center).

(b) The ratio ϕ_m/ϕ_o (maximal fluorescence over initial fluorescence). As we shall see, it depends too on the transfer efficiency and on the size of the photosynthetic unit. This ratio can be obtained from a steady-state experiment too, where ϕ_o is given by the fluorescence at low light intensity (without inhibitor) and ϕ_m is obtained by reducing the primary acceptor through the addition of sodium dithionite.

(c) The shape of the induction kinetics which gives information as to the connection between photosynthetic units. If the units are separate (i.e, each center has its own harvesting antenna) the curves should be exponential, whereas if a common array of pigments feeds energy into several centers the shape will be sigmoidal because of the increase of the average harvesting antenna per center as more centers become closed.

In their study of the development of photosynthetic units during the greening of a *Chlorella vulgaris* mutant, Dubertret and Joliot [19] showed that a decrease of the half-time of fluorescence rise is observed, followed by an evolution to sigmoidal shape. Assuming that the rate of transfers within the antenna was a constant they concluded that the first stage of greening was characterized by an increase in the size of separate units (attributed to the sole addition of chlorophyll *b*) and that the connection between units takes place at a later stage.

In order to discriminate between the role of the rate of transfer within the harvesting antenna and that of the size of the unit, we now examine a simple theoretical model. We assume separate photosynthetic units, each one including one center and n harvesting chlorophylls. The rate of photon absorption by such a unit is $n\sigma I$, where I is the light intensity and σ the absorption cross section of one chlorophyll. Absorption of a photon gives rise to an exciton which has, per unit of time, a probability, k_d to decay non radiatively, k_f to decay through fluorescence and k_t/n to be trapped by the center. k_t can be understood as a rate of exciton transfer from a chlorophyll to another, with a probability $1/n$ that the latter is a center. This is of course oversimplified, but we still suggest that k_t has roughly the meaning of an efficiency of transfer within the antenna.

The fluorescence rise under continuous illumination is exponential: $\phi_{(t)} = \phi_m - (\phi_m - \phi_o)e^{-pt}$, where p is the probability that a center becomes closed by exciton trapping per unit time. This is given by:

$$p = n\sigma I \frac{k_t/n}{k_d + k_f + k_t/n}$$

while $\phi_o = \dfrac{k_f}{k_d + k_f + k_t/n}$ and $\phi_m = \dfrac{k_f}{k_d + k_f}$.

p can be rewritten as:

$$p = n\sigma I \frac{k_t/n}{k_d + k_f} \frac{\phi_o}{\phi_m}$$

therefore

$$p \frac{\phi_m}{\phi_o} = k_t \frac{\sigma I}{k_d + k_f} \tag{1}$$

The left side of Eqn. 1 is the product of two experimentally determined quantities, p, which is inversely proportional the half-rise time, and the ratio ϕ_m/ϕ_o. It is proportional to k_t if the term $\sigma I/(k_f + k_d)$ is constant. Actually, I is kept experimentally constant, and it seems reasonable to assume that the σ, k_d and k_f of membrane-imbedded bacteriochlorophyll are fairly constant. Hence changes of the product $(t_{1/2}^{-1} \cdot (\phi_m/\phi_o))$ reflect changes of k_t, that is of the rate of exciton transfer within the antenna.

A more realistic expression for k_t can be obtained by recognizing that closed centers still trap excitation, with an efficiency $\alpha < 1$ compared to open centers. ϕ_m becomes:

$$\frac{k_f}{k_d + k_f + \alpha(k_t/n)}$$

and k_t is now proportional to

$$p \frac{\phi_m}{\phi_o} \frac{1}{1 - \alpha(\phi_m/\phi_o)}.$$

ϕ_m/ϕ_o cannot be greater than $1/\alpha$: therefore, as we get a maximum value close to 3 for ϕ_m/ϕ_o (see Tables I and II), one has $0 \leqslant \alpha < 0.33$. In the following we use the basic model with $\alpha = 0$. The consequences of a non-zero value of α are examined in the Discussion.

2. *Experimental conditions.* The experimental set-up is diagrammed in Fig. 1. Gallium arsenide diodes are used as a light source. An advantage in using these arises from their very short switching time (about 10 ns). In order to obtain a sufficient light flux (around 10 mW per 25 mm^2), 8 diodes are powered in series by a pulse generator, and the light is collected by an 8-inputs light pipe. The diodes are immersed in liquid nitrogen to improve the light yield. The emission band is centered around 640 nm, and an anticaloric filter is used to cut off the emission tail which spans beyond 750 nm. The sample cell has a square section (5 mm optical path) and fluorescence is collected at 90° C by a Y-shaped light pipe. The analyze filter cuts off wavelengths shorter than 850 nm so that the whole fluorescence emission spectrum is detected, except the 790 nm fluorescence which is emitted during the first pigmentation steps by an unbound form of bacteriochlorophyll(ide) [8,20]. The light pulse (1 s duration) is driven by the current pulse of the generator. A 1 ms time constant has been used in the kinetics recording (the half-time of the fluorescence kinetics is around 40 ms). In order to improve the signal to noise ratio, up to 9 kinetics (with a fresh dark-adapted sample each time) are averaged.

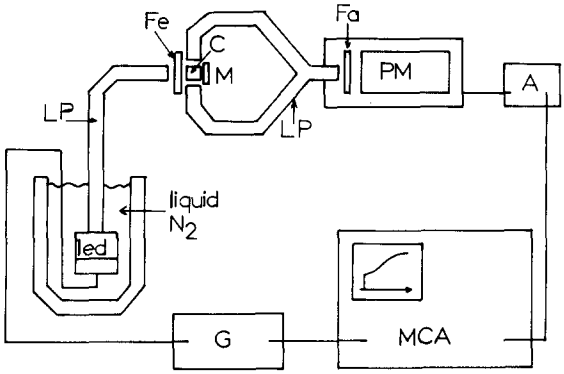

Fig. 1. Schematic diagram of the set up for fluorescence kinetics recording. G, current pulse generator (Tekelek TE 10); LED, light emitting diodes; LP, light pipe; Fe, anticaloric filter, MTO TA_2; C, sample cuvette; M, mirror; Fa, analyze filter, Wratten 87 C; PM, photomultiplier tube, 56 CVP Radiotechnique with a S 1 type cathode; A, fast current-voltage converter; MCA, multichannel analyzer Northern Tracor 575 A.

Results

Photosynthetic unit formation

In their study of the organization of pigments after semi-aerobic transfer of priorily depigmented cells, Cellarius and Peters [8] monitored the following parameters: the ratio of fluorescence yields before and after sodium dithionite (ϕ_o/ϕ_m) the ratio of in vivo fluorescence to fluorescence after pigment solubilization (relative fluorescence yield in vivo) and the ratio of fluorescence intensities when the exciting light is absorbed by carotenes or by bacteriochlorophyll. While the latter parameter gives information about the efficiency of carotene-bacteriochlorophyll transfers, the others depend on the relative amount of functional centers and on the efficiency of transfer within the bacteriochlorophyll antenna (the factor k_t defined in Material and Methods). As will be shown later, this efficiency rapidly reaches a constant value, so that ϕ_o/ϕ_m is then primarily monitoring the size of the photosynthetic unit. After 60—70 min of semi-aerobic growth, the three parameters reach plateau values, indicating an organization of pigments in functional photosynthetic units.

We undertook similar experiments with the "Phofil" mutant with the wild type as a control. The results are plotted in Fig. 2 as a function of the amount of bacteriochlorophyll per cell. The data of Fig. 2a (wild type) are similar to that of Cellarius and Peters, except for different plateau values which may be due to the use of a different fluorimetry set-up and of a different bacterial strain. The plateau is reached when the amount of bacteriochlorophyll molecules per cell is about $2 \cdot 10^5$ and spreads over to $6 \cdot 10^5$. The variations occuring after the plateau will be discussed later.

The data with the "Phofil" mutant (Fig. 2b) are similar to that of the wild type as to the plateau values of the parameters, and as to the position of the right boundary of the plateau ($6—8 \cdot 10^5$ bacteriochlorophyll molecules per cell). However, the cell pigmentation level where the constant values are reached (left boundary of the plateau) varies according to the physiological

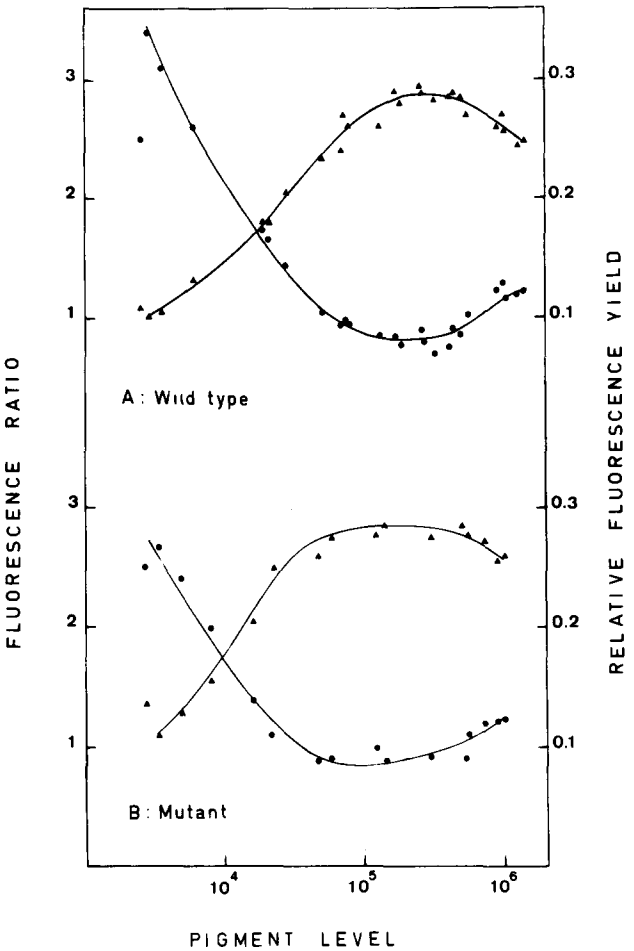

Fig. 2. Fluorescence data from repigmenting cells of parent (top) and mutant (bottom) strains. ▲——▲, (scale on the left side) ratio (ϕ_m/ϕ_o) of fluorescence intensity after dithionite addition to that before addition. Fluorescence is excited at 590 nm and detected at 885 nm; ●——●, (scale on the right) ratio of fluorescence intensity at 885 nm in vivo to that a methanol extract at 785 nm. Pigment level, number of bacteriochlorophyll molecules per cell (log scale).

TABLE I

BACTERIOCHLOROPHYLL CONTENT AND FLUORESCENCE INDUCTION DATA OF MEMBRANE EXTRACTS OF WILD TYPE *RPS. SPHAEROIDES* AND OF THREE INOCULA OF THE "PHOFIL" MUTANT

The samples were collected at the beginning of the plateau phase. Fluorescence induction curves were exponential with the four samples.

	Mutant "Phofil"			Wild type
Bacteriochlorophyll content (10^4 molecules per cell)	3	10	13.5	22
Half-rise time of fluorescence (ms)	42	39.5	41	41
Fluorescence ratio ϕ_m/ϕ_o	2.94	2.85	3	2.92

state of the mutant cells. It is the same as that of the wild type (about $2 \cdot 10^5$ bacteriochlorophylls per cell) in the case of the adapted mutant, whereas it is significantly smaller for non-adapted cells. The lowest pigmentation level we observed at the beginning of the plateau was 10^4 molecules of bacteriochlorophyll per cell.

Further information on the photosynthetic units of the wild type and of the mutant at the beginning of the plateau was obtained through fluorescence induction experiments. As the cell pigmentation is low at this stage it is necessary to isolate the membranes in order to minimize light scattering. In Table I are shown the results obtained with the wild type and three inocula of the mutant at various adaptation states. The data show that the photosynthetic units in the four cases are essentially similar: both the ratio ϕ_m/ϕ_o and the half-rise time of the induction kinetics are constant within experimental errors (and therefore so is the product $(t_{1/2}^{-1} \cdot (\phi_m/\phi_o))$ which, as explained in Material and Methods expressed the efficiency of transfer within the units). The kinetics are well fitted by a single exponential which shows that no significant transfer between units occurs. Furthermore, the absorption spectra of the four membrane preparations are identical. As shown in Fig. 3, the light harvesting *B*-850 is in a minority in this stage. A direct estimation of the amount of reaction center bacteriochlorophylls was made by measuring the bleaching at 870 nm under saturating infrared illumination. The concentration of centers was computed using an extinction coefficient of 116 $mM^{-1} \cdot cm^{-1}$ [16]. The amount

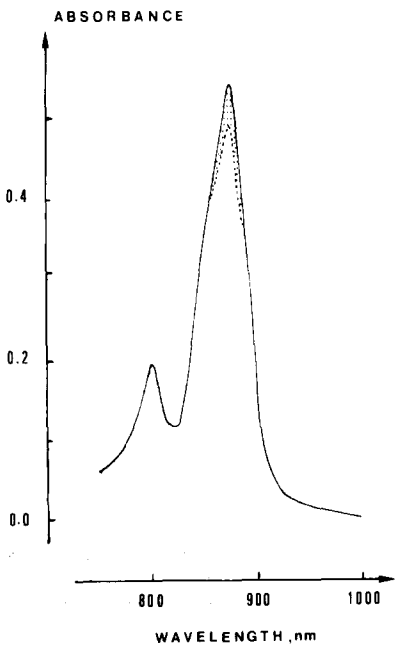

Fig. 3. Absorption spectrum of a membrane extract of *Rps. sphaeroides* (wild type). The membranes were isolated from repigmenting cells when the ratio ϕ_m/ϕ_o reaches its maximum (see Fig. 2. The relative fluorescence yield in vivo is then minimum). The pigment level was then $2.2 \cdot 10^5$ bacteriochlorophyll molecules per cell. The dotted area shows the bleaching at 870 nm observed in the IR 2 mode of a Cary 14 (strong white illumination), spectrometer.

of *B*-875 and *B*-850 were determined from the spectra, correcting for their respective contributions (and that of the centers) with the assumption of symmetrical absorption bands. A ratio *B*-875/*B*-850 of 2.33 was obtained for the wild type, and 2.65 for the less pigmented of the three mutant preparations, while the relative amount of centers *P*-870/(*B*-875 + *B*-850) was 1/37 for the wild type and 1/31 for the mutant.

Therefore, there is no significant difference in the photosynthetic units of the mutant and of the wild type and that the low rate of bacteriochlorophyll synthesis in the unadapted "Phofil" mutant [11] means a low synthesis of otherwise normal units.

Photosynthetic unit development

We now examine the evolution of the spectral and fluorescence characteristics of the wild type *Rps. sphaeroides* Y during the repigmentation process. In addition to Fig. 2A, Table II gives further information obtained with membrane preparations from cells harvested at various stages of repigmentation. Before membrane extraction, the ratio ϕ_m/ϕ_o (using sodium dithionite to obtain ϕ_m) was measured in whole cells. The values obtained are consistent with those of Fig. 2A with a plateau extending from $2 \cdot 10^5$ to $5\text{—}6 \cdot 10^5$ molecules of bacteriochlorophyll per cell (i.e., from 1.5 to 3 h, see Fig. 5, top). The ratio then decreases down to values similar to those of control cultures or incubating in Erlenmeyer flasks on a low speed shaker).

In Table II are given the values of the product $(t_{1/2}^{-1} \cdot (\phi_m/\phi_o))$ where $t_{1/2}$ is the half-rise time of the fluorescence of the membrane extracts at a fixed intensity and ϕ_m/ϕ_o the fluorescence ratio obtained in a dithionite experiment. As explained in Material and Methods, this product is proportional to k_t which is (roughly) a rate constant for transfers within the antenna. It can be seen that

TABLE II

BACTERIOCHLOROPHYLL CONTENT AND FLUORESCENCE INDUCTION DATA OF MEMBRANE EXTRACTS OF REPIGMENTING *RPS. SPHAEROIDES*

Absorbance at 850 and 875 nm corrected by the method of Crounse et al. [14]. Controls were made on chromatophores isolated (1) from semi-aerobic culture shaked in Erlenmeyer flask and (2) from photosynthetic culture. The fluorescence induction kinetics were well fitted by a single exponential in the 6 first samples (45—240 min). The 350 and 470 min samples and both control samples yielded sigmoidal induction curves.

	Time after decreasing oxygen pressure (min)								Control	
	45	75	90	170	200	240	350	470	(1)	(2)
Pigment level (10^4 molecules per cell)	2.5	13.5	22	50	67	80	125	135	250	390
ϕ_m/ϕ_o *	1.75	2.62	2.92	2.92	2.75	2.65	2.50	2.45	2.38	2.31
Half-rise time of fluorescence (ms)	47	46	41	41	38	36	33	33	34	33
$(t_{1/2}^{-1} \cdot \phi_m/\phi_o) \cdot 10^2$ **	3.72	5.69	7.12	7.12	7.24	7.36	7.58	7.42	7	7
A_{875}/A_{850}	4.7	3.4	2.33	1.29	0.93	0.91	0.73	0.62	0.53	0.45

* Fluorescence ratio at 885 nm after to before dithionite addition.

** The product $(t_{1/2}^{-1} \cdot \phi_m/\phi_o)$ is proportional to k_t (efficiency of energy transfer in the antenna, see Material and Methods).

k_t first rises before the plateau is reached and then remains constant, even after the plateau stage. This allows a general interpretation of the evolution of parameters displayed in Fig. 2, Table II or Fig. 5 (top). Before the plateau is reached, the pigments are, on the average, poorly connected to each other. As the degree of connection increases, so does k_t (Table II and Fig. 5, top), and so does the efficiency of quenching by the centers as shown by the increase in ϕ_m/ϕ_o or by the decrease of the in vivo/in vitro fluorescence ratio. Once the plateau is reached, k_t becomes constant so that the primary cause for the evolution of the fluorescence parameters is the increase of the photosynthetic unit size (the eventuality of a decrease of k_t, which will be envisaged in the Discussion, would only strengthen this assertion). At the beginning of the plateau, we found out that there were about 30 bacteriochlorophylls per center. As the unit size increases the half-rise time of the induction kinetic decreases and so does the ϕ_m/ϕ_o ratio. This appears in the simple model we examined in Material and Methods where the half-rise time of fluorescence was proportional to: $1/n + (k_d + k_f)/k_t$, and where the ratio ϕ_m/ϕ_o was equal to: $1 + k_t/(k_d + k_f)\ 1/n$ (n is the size of the unit, i.e., the number of harvesting bacteriochlorophylls per center). During the plateau phase the half rise-time of fluorescence remains constant (41 ms), whereas the pigment level trebles from $2 \cdot 10^5$ up to $6 \cdot 10^5$ molecules of bacteriochlorophyl per cell. In the later phase during which the pigmentation level doubles, the half rise-time decreases down to 33 ms, a value identical to that obtained with the photosynthetic culture control. The shape of the kinetics changes too, becoming sigmoidal when the half-rise-time approaches its final value of 33 ms (Fig. 4). The sigmoidal character then becomes more marked while the half rise-time

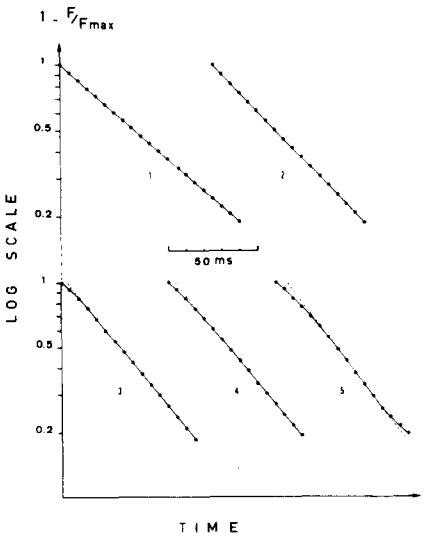

Fig. 4. Semi-log plot of fluorescence induction kinetics in the presence of o-phenanthroline (1 mM), from membrane extracts of *Rps. sphaeroides* (wild type) isolated at various stages during the repigmentation. Samples 1, 2, 3 and 4 correspond to, respectively, 90, 240, 350 and 470 min growth in semi-aerobic (95% N_2/5% O_2) conditions, and sample 5 was a photosynthetic culture. The half-rise time of fluorescence are 41 and 36 ms, respectively, for curves 1 and 2, 33 ms for curves 3, 4 and 5.

remains at 33 ms, indicating that the degree of connection between units increases without a change of the effective antenna area per center.

Aagaard and Sistrom [1] and more recently, Niederman et al. [3] showed that the ratio of the two types of light harvesting complexes varies according to the culture conditions, especially that *B*-875 was preferentially synthesized at the beginning of the repigmentation process. Our data are consistent with this conclusion as indicated by the variations of the ratio *A*-875/*B*-850 in Table II and those of the amounts per cell of both complexes plotted in Fig. 5 (bottom). Aagaard and Sistrom [1] claimed that there was a fixed stoechiometry between reaction center and *B*-875. At the beginning of the plateau we measured a ratio of 1 center per 37 light-harvesting bacteriochlorophylls (*B*-850 + *B*-875). Taking the values of the amount of the two light-harvesting forms at this stage, we get a stoichiometry of 26 *B*-875 per center, which is

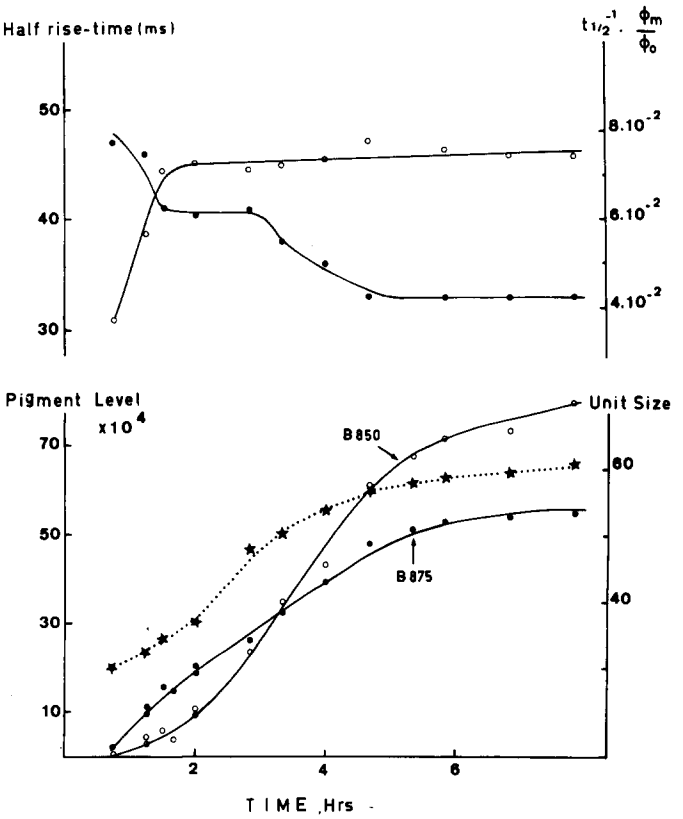

Fig. 5. Bottom: time course of *B*-850 (○) and *B*-875 (●) synthesis in repigmenting *Rps. sphaeroides* (wild type). Vertical scale, number of pigments per cell. The bacteriochlorophyll content per cell was computed from measurements with an acetone/methanol (7 : 2, v/v) extract. The ratio *B*-850/*B*-875 was computed from absorption spectra of membrane extracts, using the method of Crounse [14] and the extinction coefficients given by Sistrom [15]. The absorption of *B*-875 was further corrected for the contribution of *P*-870. ★·····★, Computed size of the photosynthetic units assuming a fixed ratio *B*-875/*P*-870 (see text). Top: time course of the half rise-time of fluorescence kinetics (●), and of the product ($t_{1/2}^{-1} \cdot \phi_m/\phi_o$) (○) with is roughly proportional to the rate of exciton transfer within the antenna (see Material and Methods).

close to the value of 20—25 given by Aagaard and Sistrom [1]. From this stoechiometry and the data of Fig. 5 we can compute the size of the unit at any time by adding to the basic unit of 26 *B*-875 the amount of *B*-850. The result was plotted in Fig. 5 (bottom). After 7 h of repigmentation, the unit size would be 62 bacteriochlorophylls/center.

Discussion

As noted previously [11], the mutational event in the "Phofil" mutant does not bear directly on the building of the photosynthetic apparatus but rather on the mode of growth and enlargement of the various membrane layers of the cell envelope. This unbalanced growth leads to a restriction of the integration rate of synthesized pigments into the inner membrane. The experiments reported in the previous section show that this low rate has no consequence on the functionality of the units but means rather a low rate in the building of otherwise normal units. This is an argument against the hypothesis of a "statistical" building process of the photosynthetic apparatus by random integration of pigments into the membrane. An estimate of the total membrane surface area per cell, prior to the formation of internal vesicles, can be obtained from cell dimensions. From membrane area (assuming an average cell length of 2 μm and 0.5 μm diameter) and cellular amount of bacteriochlorophyll, we estimate the available membrane area per pigment molecule at the beginning of the plateau (Fig. 2) 1600 Å^2 for the wild type and 35000 Å^2 for the less pigmented, unadapted mutant. This corresponds to average inter-molecular distances of 40 Å for the wild type and 200 Å for the mutant. This latter value is definitely inconsistent with the random integration model and the requirement of small distances for efficient transfer to occur. On the other hand, one might think of units formation as a simple agregation process of the pigments complexes and centers within the membranes. However, the exponental kinetics of fluorescence induction show that the units remain separate until late stages of the pigmentation process. Put together, this strongly argues in favor of a formation of photosynthetic units through integration of pigments into discrete membrane sites. Our data, however, do not allow an estimation of the degree of order in the location of these sites of initiation.

The results reported here on the repigmentation process of wild type *Rps. sphaeroides* agree with the hypothesis of sequential integration of the various bacteriochlorophyll holochromes. A major aim of this study was to correlate this sequential mode of assembly with the formation and development of photosynthetic units. At the beginning (before the plateau phase in Fig. 2) the pigments are located comparatively far from each other as shown by the low rate ($t_{1/2}^{-1} \cdot \phi_m/\phi_o \propto k_t$ in Table II) and the low efficiency of photochemical trapping as shown by the high values of the relative fluorescence yield in vivo (Fig. 2). It must be pointed out that this is only average information which does not necessarily mean that all pigments have the same low efficiency of transfer, but rather that a significant fraction of the pigments is not yet incorporated in functional units, while another fraction is. During this phase, the *B*-875 synthesis is greater than that of *B*-850 (Fig. 5 and Table II).

The low ϕ_m/ϕ_o ratio at this stage does not monitor the size of the antenna but is mainly due to the low transfer efficiency. At the end of this phase, the pigments are now organized in functional photosynthetic units where the various parameters we have studied take on optimal values: the transfer rate (k_t) reaches its maximal value, so does the trapping efficiency (ϕ_m/ϕ_o maximum, relative fluorescence yield in vivo minimum). Actually, the occurrence of a plateau of the fluorescence parameters while the *B*-850/*B*-875 ratio is increasing should be interpreted as an averaging over different processes: while the existing units increase in size through the preferential incorporation of *B*-850, the building of new units continues.

The further development of the photosynthetic apparatus proceeds then mostly by the progressive addition of *B*-850 (Fig. 5). This entails an increase in the size of photosynthetic units as shown by the fluorescence parameters: decrease of $t_{1/2}$, decrease of ϕ_m/ϕ_o. The unit size can be estimated directly from the *B*-850—*B*-875 amounts assuming a fixed stoechiometry *B*-875—*P*-870 (Fig. 5, bottom). However, a difficulty arises from the fact that after 5 h repigmentation the half rise-time of fluorescence kinetics remains constant (33 ms) (Fig. 5, top) while the unit size computed from the pigment composition still increases slowly. Moreover, the shape of the fluorescence induction (Fig. 4) shows that transfer between units is becoming significant after 6 h repigmentation, which should further increase the effective unit size. The contradiction is more conspicuous with the photosynthetic culture data which yielded a unit size of 83 bacteriochlorophylls/center, whereas the half rise-time was still 33 ms. An explanation may be that a lower transfer efficiency of the harvesting pigments somehow compensates for the antenna enlargement. This is not confirmed by the behaviour of parameter k_t (Table II and Fig. 5), which remains constant. However, the picture is changed if one takes into account a non-zero probability of trapping (α) by closed centers. As mentioned in Material and Methods, the expression we used for k_t must be divided by $(1 - (\phi_m/\phi_o))$. That $\alpha > 0$ is indicated by three lines of evidence: the fluorescence ratio ϕ_m/ϕ_o is by far smaller than would be expected from the photochemical quantum yield (i), mutants which lack photochemical centers have a higher fluorescence yield and lifetime than the wild type in the closed centers state [21] (ii), and the formation of a short-lived radical pair in closed centers has been demonstrated [22] (iii). We have no sufficient information to assign a precise value to α, but an upper limit of 0.33 is implied from the higher values we find for ϕ_m/ϕ_o ($\simeq$3). Taking $\alpha = 0.1$ does not change quantitatively the behaviour of k_t described in the text. However, taking higher values such as $\alpha = 0.3$ has the following consequences: k_t still rises steeply during the first 90 min of pigmentation, reaches a maximum between 90 and 170 min, and then decreases asymptotically to an intermediate value (that of the photosynthetic culture control). Therefore, we cannot exclude a decrease in transfer efficiency after the plateau phase.

Obviously, this point needs further investigation, for it is difficult to understand why an increase in unit size should take place, if not for an improved light collection efficiency. An alternative hypothesis would be that after 5 h repigmentation there is not really a significant increase in the unit size, i.e., that the fixed stoechiometry *B*-875/*P*-870 is not always true and that under certain conditions the synthesis of *P*-870 may not parallel that of *B*-875.

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