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Changes in the content of pigment-protein complexes in *Rhodobacter sphaeroides* forma sp. *denitrificans* grown under photosynthetic and photo-denitrifying conditions

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Changes in the relative content of pigment-protein complexes, RC-B880 and B800-850, were studied in membranes of *Rhodobacter sphaeroides* forma sp. *denitrificans* cultured under various anaerobic conditions. The content of each pigment-protein complex was determined by the decomposition of the absorption spectra of membranes in the near-infrared region into the spectra of RC-B880 and B800-850. The standard spectrum of each complex in the membranes was obtained using two absorption spectra of membranes with different ratios of the complexes by eliminating the spectrum of first one than the other complex. Spectra composed from the two standard spectra were in good agreement with original membrane spectra after subtraction of the contribution of scattering in various membrane samples. Bacteriochlorophyll (BChl) content in the membrane was dependent on the light intensity during growth. The relation between the total BChl content in the membrane and BChl content in the RC-B880 and B800-850 complex was linear above 15 nmol BChl per mg membrane protein, regardless of the culturel conditions, photosynthetic or photo-denitrifying. The linear relationship reached a point where all BChl molecules were contained in RC-B880 at 13 nmol BChl per mg membrane protein. This means that only RC-B880 would be synthesized below the threshold, and above the threshold additional BChl was distributed between RC-B880 and B800-850 in a constant ratio (1:5.7). The results suggest that the syntheses of B800-850 and RC-B880 are not regulated independently.

Introduction

Bacteriochlorophyll (BChl) molecules are present in pigment-protein complexes in the membranes of purple photosynthetic bacteria [1–3]. Most BChl molecules are present in light harvesting (LH) pigment protein complexes and the rest in reaction center (RC) complexes [4–6]. In the absorption spectrum of photosynthetic membranes from *Rhodobacter sphaeroides*, LH BChl exhibits some absorption peaks in the near-infrared region. These peaks have been attributed to three BChl forms, B800, B850 and B880 *, which

Abbreviations: RC, reaction center; BChl, bacteriochlorophyll; Mops, 4-morpholinepropanesulphonic acid; LH, light harvesting.

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* The in vivo absorption maximum of an LH complex which was referred to as B870, B875 or B880, was at 880 nm in the present organism and is referred to as B880 in this paper.

are contained in two kinds of complexes, namely the B880 complex (LH I complex) and B800-850 complex (LH II complex). B880 complexes have been shown to be associated with RC complex in vivo to make a super-complex, RC-B880 complex, in a constant ratio (RC : B880 is about 1 : 6 as BChl) [1,7–9].

It has been known that the absorption spectrum of membranes varies depending on growth conditions, such as concentrations of O_2 or light intensity [5,10–16]. In *Rb. sphaeroides,* the variation can be ascribed to the changes of the content of RC-B880 complexes relative to B800-850 complexes in the membranes. In spite of many studies on the control mechanisms for the synthesis of B800-850 and B880, attempts for precise determination of the contents or the ratio of the two complexes have rarely been made. Aagaard and Sistrom [7] and Sistrom [9] estimated the relative content of B880 and B850 by solving equations derived from some assumptions. Their results were highly significant in suggesting the constant ratio of B880 to RC. They also speculated the independent control of the synthesis of B880 and B850. The methods, however, do not seem to be adequate to estimate the precise ratio of B880 to B850.

It has been reported that the membrane absorption spectra were well expressed as the sum of spectra of isolated complexes, RC-B890, B800-820 and B800-850, in *Chromatium vinosum* [17], and RC-B870 and B800-850, in *Rhodopseudomonas palustris* [18]. In *Rb. sphaeroides*, Clayton and Clayton obtained an in vivo absorption spectrum for the B800-850 complex by subtracting the spectrum of the isolated B880 complex from the spectrum of chromatophores [19], and Meinhardt et al. simulated membrane spectra using the spectra of B880-free and B800-850-free mutants [20]. The absorption peaks of isolated complexes [19] or mutant membranes [20], however, were shifted slightly from those of membranes from wild type strains and the contribution of the absorption by RC was not appropriately considered in those works. In addition, only a little attention was given to the effect of scattering, which becomes significant in the membranes with low pigment content.

Rb. sphaeroides forma sp. *denitrificans* can grow by photosynthesis, respiration or denitrification [21]. The LH pigment system [22] and alteration in the BChl content in cells grown under various conditions of light intensity or oxygen concentration are similar to those of *Rb. sphaeroides* strains which lack the property of denitrification. Michalski et al. [23] suggested the loss of B880 complex under the denitrifying conditions as the effect of nitrite, but our preliminary observations were inconsistent with their postulation. In this study we analyzed the near-infrared absorption spectra of membrane preparations in order to study the quantitative changes of RC-B880 and B800-850 complexes when the cells were grown in various light intensities in the presence or absence of nitrate. The regulation of the synthesis (and possible decomposition) of pigment-protein complexes under photo-denitrifying conditions was shown to be essentially the same as that under photosynthetic conditions. Two phases of the anaerobic regulation of the synthesis of the complexes was revealed under both conditions.

Materials and Methods

The denitrifying phototrophic bacterium, *Rb. sphaeroides* forma sp. *denitrificans* IL106 was used. Cells were cultured anaerobically at 30 °C under illumination with tungsten lamps in a medium [21] supplemented with 0.3% sodium malate as the carbon source and, in the case of denitrifying cultures, with 0.2% KNO_3 in addition. Light intensity was varied by alteration of the distance from an incandescent lamp (1 W/m^2 corresponding to about 240 lux).

Cells harvested in the early stationary phase were washed and suspended in 10 mM Mops-Na buffer (pH 7.0) containing 100 mM KCl. The cell suspensions were sonicated at 20 kHz (140 W) for 3 min, and membrane fractions were obtained by subjecting the sonicate to differential centrifugation between 17 000 × *g* for 15 min and 218 000 × *g* for 1 h.

The absorption spectra of the membrane fractions were measured with a Hitachi 220A or Shimadzu UV-160 spectrophotometer. The absorbance values from 700 to 900 nm or to 1000 nm were transferred at 1 nm intervals to a per-

sonal computer NEC PC-8001 and recorded on a floppy disc.

Curve analyses of absorption spectra were performed with a personal computer Fujitsu FM-16β. The membrane spectrum was assumed to be composed of the spectra of RC-B880 complexes, B800-850 complexes and scatterings by small and large particles. The in vivo spectra of these four components were determined as follows. The scattering by large particles was assumed to be independent of wavelength. The scattering by small particles was assumed to follow Rayleigh scattering and, therefore, to be inversely proportional to the biquadratic of wavelengths. Spectra of RC-B880 and B800-850 for initial calculation were taken from the spectra of the isolated complex reported by Clayton and Clayton [19]. From the spectra of these four components, the values of absorbance at 700, 850, 880 and 1000 nm were used as initial coefficients in four simultaneous equations, which were similar to those shown in 'Results', except that the coefficients for the pigment absorbances were different from the final values. The contribution of each component in a membrane preparation was determined by solving the equations. Then, the scattering components were subtracted from the membrane spectrum so that the spectra caused by coloured components, RC-B880 and B800-850, were retained (the spectrum caused by the coloured components is referred to below as the scattering-subtracted membrane spectrum). Two scattering-subtracted membrane spectra, in which the relative content of the two complexes was different, were chosen and the spectrum of one kind of complex was subtracted to leave the other, using the results of the calculation described above. The spectrum left was a tentative in vivo spectrum of RC-B880 complexes or B800-850 complexes. From these tentative spectra, the absorbances at 700, 850, 880 and 1000 nm were taken and put into the simultaneous equations again as the coefficients, instead of the initial values from the spectra of the isolated complexes. These processes were repeated until the coefficients converged within a difference of 1% compared with the previous coefficients. The purity of each decomposed spectrum during the repeating process was examined by taking the secondary derivative of the spectrum. When an overlapping of the spectrum of the other complex was obvious from the spectral shape around 850 and 880 nm, working coefficients were modified a little (1–2%) to decrease the overlapping effect. In order to calculate separately in vivo absorption coefficients of BChl in RC-B880 and B800-850, BChl concentrations of two different membrane preparations were determined after acetone-methanol extraction [25], and the following simultaneous equations were solved.

$$A_1(\text{RC-B880})/x + A_1(\text{B800-850})/y = C_1$$

$$A_2(\text{RC-B880})/x + A_2(\text{B800-850})/y = C_2$$

where A(RC-B880) and A(B800-850) are absorbances at a certain wavelength of the decomposed spectra of RC-B880 and B800-850 in each membrane preparation, C is the millimolar concentration of BChl determined after acetone-methanol extraction, and x and y are millimolar absorption coefficients of BChl in RC-B880 and B800-850, respectively, at the wavelength. Subscript numbers indicate two different membrane preparations.

The determination of protein concentrations was performed by the method of Lowry et al. [24] (with a standard of bovine serum albumin) after heating at 95°C for 5 min in 0.1 M NaOH containing 2% Na_2CO_3.

Results

The membrane spectrum was decomposed into the spectra of RC-B880 and B800-850 complexes after subtraction of the scattering effect. The in vivo standard spectra of RC-B880 and B800-850 complexes (Fig. 1a) were obtained from a couple of membrane spectra which differed in the relative content of the two complexes. The shapes of the decomposed spectra were practically independent of the membrane preparation used for the decomposition, except that a small (less than 1 nm) blue shift of the peak in the B800-850 complex from high light culture was observed (data not shown). In order to examine the purity of the in vivo standard spectra obtained, secondary derivatives were taken for the decomposed spectra (Fig. 1b). Negative peaks in the secondary derivatives corre-

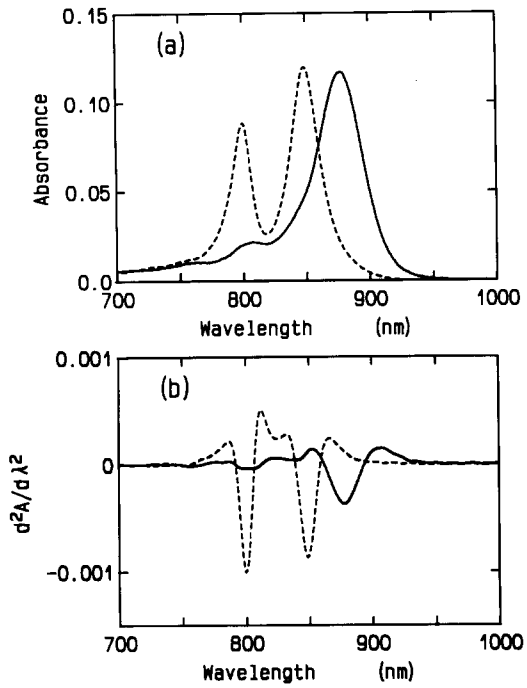

Fig. 1. (a) The in vivo standard spectra of RC-B880 (solid line) and B800-850 (broken line) in membrane fractions from *Rb. sphaeroides* forma sp. *denitrificans*. Each spectrum was normalized to 1 mM BChl. The calculation procedure is described in Materials and Methods. (b) Secondary derivatives of the in vivo standard spectra. Solid line, RC-B880 complex; broken line, B800-850 complex.

spond to absorption bands in the original spectra. The negative peak at 850 nm was negligible in the secondary derivative of the absorption spectrum for RC-B880 (solid line) and no peaks around 880 nm were detected in that of B800-850 (dashed line). This shows that each of the decomposed spectra contained little contamination caused by the other complex.

TABLE I

ABSORPTION COEFFICIENTS FOR BChl IN RC-B880 AND B800-850 IN THE MEMBRANES OF *RB. SPHAEROIDES*

Wavelength (nm)	Absorption coefficient ($mM^{-1} \cdot cm^{-1}$)	
	RC-B880	B800-850
700	6	5
850	49	130
880	116	19
1000	0	0

Absorption coefficients for BChl in the RC-B880 and B800-850 complexes in vivo are shown in Table I. The absorption coefficients at the peak wavelengths, 116 $mM^{-1} \cdot cm^{-1}$ at 880 nm in RC-B880 and 130 $mM^{-1} \cdot cm^{-1}$ at 850 nm in B800-850, were almost the same as those obtained by Clayton and Clayton [19] with isolated complexes (126 $mM^{-1} \cdot cm^{-1}$ for B880 without RC, and 132 $mM^{-1} \cdot cm^{-1}$ for B800-850).

Using the in vivo absorption coefficients, BChl concentrations in each complex were calculated from the absorbances of membrane preparations. Simultaneous equations for the BChl concentrations in the two complexes and the contributions of scattering were as follows.

$$A_{700} = 6*[\text{RC-B880}] + 5*[\text{B800-850}] + 1.00*[\text{SCS}] + [\text{SCL}]$$

$$A_{850} = 49*[\text{RC-B880}] + 130*[\text{B800-850}] + 0.46*[\text{SCS}] + [\text{SCL}]$$

$$A_{880} = 116*[\text{RC-B880}] + 19*[\text{B800-850}] + 0.40*[\text{SCS}] + [\text{SCL}]$$

$$A_{1000} = 0*[\text{RC-B880}] + 0*[\text{B800-850}] + 0.24*[\text{SCS}] + [\text{SCL}]$$

$$(A_{740} = 8*[\text{RC-B880}] + 9*[\text{B800-850}] + 0.80*[\text{SCS}] + [\text{SCL}])$$

where [RC-B880] and [B800-850] are BChl concentrations (mM) of RC-B880 and B800-850 complexes, and [SCS] and [SCL] are Rayleigh scattering expressed as absorbances at 700 nm and wavelength-independent scattering, respectively. (The fifth equation in parentheses was used instead of the fourth equation when absorbance values at 1000 nm were not available by using the Hitachi spectrophotometer.) The concentration of BChl in each complex in any membrane preparation can be calculated by solving the simultaneous equations. It is known that the scattering component by small particles is not linearly dependent on the membrane concentration when the multiple scattering is significant due to high concentration. Probably for this reason, the above equations were not applicable for dense membrane suspensions in which the absorbance at 700 nm was more than 0.7. Below this value, the amplitude of scattering was proportional to the membrane concentration. The present measurements were carried out in this concentration range.

In order to evaluate the BChl concentrations calculated, the in vivo standard spectra for RC-

B880 and B800-850 complexes were multiplied by the BChl concentrations obtained and added up (Fig. 2a). The added spectrum was termed as a composite spectrum. The difference between the scattering-subtracted membrane spectrum and the composite spectrum was used to evaluate the reliability of this method to quantify the pigment-protein complexes (Fig. 2b, ordinate scale multiplied by 10). In many membrane preparations from cells grown in different conditions, the scattering-subtracted membrane spectra and the corresponding composite spectra were found to be in good agreement. Standard deviation of the maximum difference (700–1000 nm) between the composite absorbances and the scattering-subtracted absorbances was 0.031 ($n = 27$) when the maximum absorbances (700–1000 nm) of scattering-subtracted spectra were normalized to 1. This good agreement confirms the validity of the above equations.

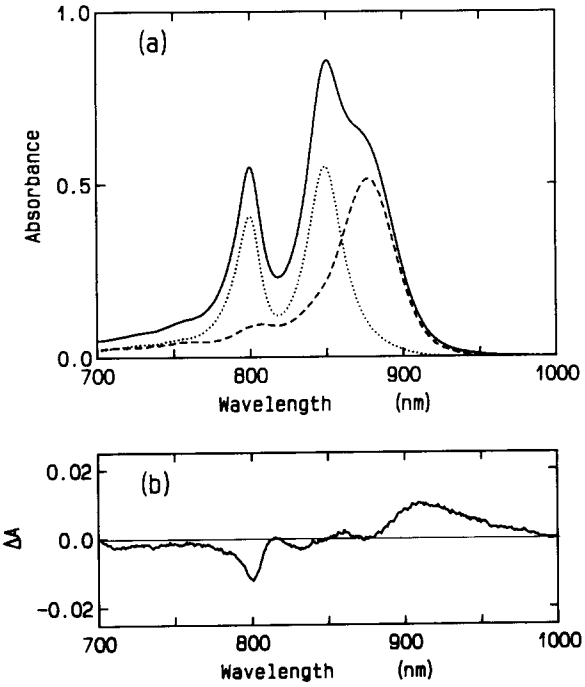

Fig. 2. (a) Decomposition of a membrane spectrum into the spectra of RC-B880 and B800-850. Solid line, in vivo spectrum of membrane fraction. The contribution of scattering has been subtracted, as described in the text. Broken line, the calculated contribution to the spectrum of RC-B880. Dotted line, the contribution to B800-850. (b) A difference spectrum between membrane and composite spectra. The composite spectrum of RC-B880 and B800-850 spectra shown in (a) was subtracted from the scattering-subtracted membrane spectrum. The ordinate scale was multiplied by a factor of 10.

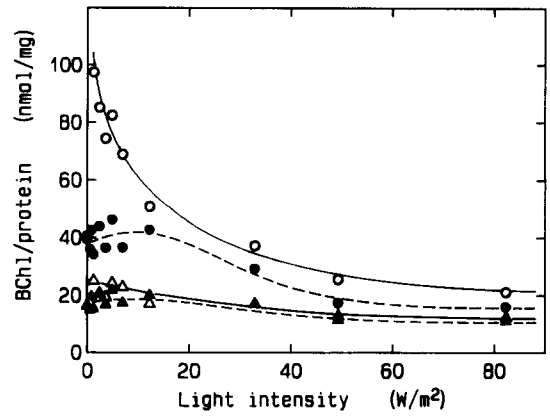

Fig. 3. Dependence of BChl content on light intensity. Open symbols (○, △) and solid lines, photosynthetic cultures ($-NO_3^-$); Closed symbols (●, ▲) and broken lines, photo-denitrifying cultures ($+NO_3^-$); ○ and ●, total BChl content in membranes; △ and ▲, BChl content in RC-B880. Other culture conditions were described in Materials and Methods. BChl concentrations were determined from membrane spectra by solving the simultaneous equations described in Results.

Fig. 3 shows the dependence of BChl concentrations (total and in RC-B880) per membrane protein on light intensity during growth. The total BChl increased sharply by lowering the light intensity from 20 W/m^2 in the photosynthetic culture, whereas the total content of BChl in the photo-denitrifying culture were almost constant at less than 20 W/m^2. In the photosynthetic cultures, no significant growth was observed below 0.8 W/m^2. The total BChl content in the photosynthetic culture was always larger than that in the photo-denitrifying culture at the same light intensity.

The BChl contents of RC-B880 and B800-850 in membranes from cells grown under various conditions were plotted against the total BChl contents to examine the relationship between total BChl biosynthesis and the BChl incorporation into each complex (Fig. 4). The relationship between the total BChl and BChl in RC-B880 (Fig. 4a) or B800-850 (Fig. 4b) follows a straight line regardless of the culture conditions, photosynthetic or

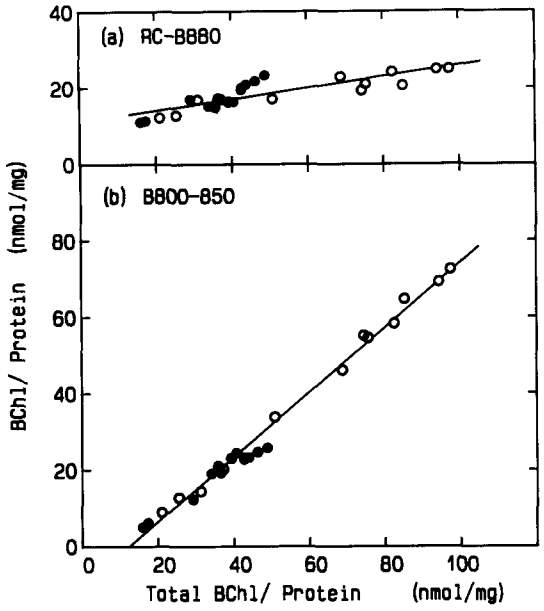

Fig. 4. Relationships between total BChl content in membranes and BChl content in RC-B880, (a) and B800-850, (b). Data presented in Fig. 3 are replotted. ○, photosynthetic cultures; ●, photo-denitrifying cultures. Solid lines were drawn by a least-squares fit.

photo-denitrifying. By a least-squares fit, the relationships were expressed as follows.

$$\text{BChl(RC-B880)} = 0.15 * \text{BChl(total)} + 11.1$$

$$\text{BChl(B800-850)} = 0.85 * \text{BChl(total)} - 11.1$$

The coefficients 0.15 and 0.85 mean that 15 and 85% of BChl increase were due to RC-B880 and B800-850, respectively, in the wide range of the specific BChl content of 15–100 nmol per mg protein. The line for B800-880 (Fig. 4b) crosses the abscissa at 13 nmol of total BChl per mg protein. This suggests that BChl molecules in the membrane would be predominantly present in RC-B880 below 13 nmol BChl per mg protein, although such preparations were not obtained in the present experiments.

Discussion

In this study, the spectral analysis was shown to be useful for the determination of the contents of the two pigment-protein complexes in membranes of *Rb. sphaeroides* forma sp. *denitrificans*. The estimation of scattering by two components, one inversely proportional to the biquadratic of wavelength and the other independent of wavelength, and the use of in vivo standard spectra, instead of the spectra of isolated complexes, have contributed to improve the accuracy of the determination. There were only slight changes observed in the spectra of RC-B880 and B800-850 complexes when the growth conditions were changed. The composite spectra based on decomposed standard spectra for the two complexes were in good agreement with the scattering-subtracted membrane spectra from cells grown under various conditions. The in vivo absorption coefficients (Table I) and the equations in Results provide a convenient and reliable method to determine the BChl concentrations in the two pigment-protein complexes in the membrane. It has been proposed that the numbers of BChl molecules in the minimal unit of each complex of RC, B880, and B800-850 are 4, 2 and 3, respectively, and that the ratio of RC to B880 in the RC-B880 complex is 1 : 12 on the unit complex basis [1–3]. Using these numbers, concentrations of each complex can also be calculated. For example, concentrations of RC can be determined as 1 : 28 of BChl concentrations of RC-B880. RC concentrations obtained by this method were in good agreement with those determined by the light-induced oxidation of $(BChl)_2$ (data not shown) using the absorption coefficients described in Ref. 26.

Michalski et al. [23] reported that denitrifying cells of *Rb. sphaeroides* forma sp. *denitrificans* were lacking in B880 (B870 in the original paper), due to nitrite formed from nitrate in the culture medium. But, in this study, the B880 component was shown to be always present under any denitrifying conditions employed. The content of RC-B880 complexes in the membranes from denitrifying cells was almost the same as that in the photosynthetic culture which showed the same specific BChl content (Fig. 4). We conclude that denitrifying conditions did not cause preferential decomposition of B880, as was postulated by Michalski et al., at least under the culture conditions we employed.

The relationship between the increase in the contents of total BChl and that in RC-B880 or B800-850 complex was found to be linear (Fig. 4).

This indicates that a mechanism is working to regulate the biosynthesis of RC-B880 and B800-850 to a constant ratio irrespective of the light intensity or the presence of nitrate. The results in Fig. 4b also indicate that below the specific BChl content of 13 nmol/mg protein, practically no B800-850 complex would be present in the membranes. Under the conditions in which more BChl was synthesized than the threshold, supplementary RC-B880 complexes and B800-850 complexes were synthesized in a constant ratio, approx. 1 : 5.7 (RC-B880 : B800-850) in molar ratio of BChl. This apparent biphasic regulation of the synthesis of the pigment-protein complexes seems to be different from previous speculations, which indicated the independent control of the synthesis of each complex [9].

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