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Determination of the bacteriochlorophyll:carotenoid ratios of the B890 antenna complex of *Rhodospirillum rubrum* and the B800–850 complex of *Rhodobacter sphaeroides*

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The ratio of bacteriochlorophyll *a* (BChl) to carotenoid in both the B890 light-harvesting pigment-protein complex of *Rhodospirillum rubrum*, strain S1, and the B800–850 complex of *Rhodobacter* (formerly called *Rhodopseudomonas*) *sphaeroides*, strain 2.4.1, has been determined by HPLC and two other methods. We report that the BChl-to-carotenoid ratio for both complexes is 2:1.

Introduction

Under anaerobic conditions light-grown purple bacteria develop photosynthetic membranes which contain a high proportion of pigment-protein complexes. Most of these are antenna complexes which harvest the incident light and funnel the resulting excitation energy to the reaction centres where it is 'trapped'. The structures of the reaction centres from *Rhodopseudomonas viridis* [1,2] and *Rhodobacter sphaeroides* R26 [3–5] have been determined by X-ray crystallography to a resolution of less than 3 Å. The structures of the light-harvesting antenna complexes are less well defined. Each complex is known to comprise oligomeric pairs of small transmembrane proteins to which BChl and carotenoid pigments are non-covalently attached [6,7]. The pigments are held in the hydrophobic interior of the membrane by specific interactions with the polypeptide side-chains, and are positioned in a way which allows efficient energy transfer between them. The transmembrane α-helical arrangement of the apoproteins is well documented [6–8] but little is known about the interaction of these proteins with their associated pigment molecules. For a complete understanding of the structure and function of antenna complexes the ratios of the individual components must first be determined. Most of the antenna complexes studied so far contain α- and β-polypeptide subunits in a ratio of 1:1 [9]. In the B890 antenna type two BChl molecules bind to each α/β-polypeptide pair [7,10], whereas three are bound in B800–850-complexes [10,11]. The ratio of BChl to carotenoid in the B890 complex of *R. rubrum* has been consistently determined to be 2:1 [9,12,13]. However, there have been conflicting reports about the ratio of the B800–850 antenna type. The ratio was originally determined in *Rb. sphaeroides* by Cogdell and Crofts to be 3 BChl *a*:1 carotenoid [14,15]. Since then the ratio

Abbreviations: BChl, bacteriochlorophyll; LDAO, lauryldimethylamine *N*-oxide; NIR, near-infra-red region.

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in the analogous B800–850 complex of *Rb. capsulatus* has been determined to be both 3:1 [16] and 2:1 [17], and Radcliffe et al. [18] have reported a ratio of 2:1 in *Rb. sphaeroides*, although in the latter case no experimental data were given. Most recently the ratio in *Rb. sphaeroides* has been determined to be 3:1 [12].

A 2:1 ratio was assumed by Kramer et al. [8] in their well-known model for the B800–850 complex of *Rb. sphaeroides*. Because of the conflict over the pigment content of this complex, we set out to obtain an unequivocal value for this ratio, with the aim of testing the validity of the stoichiometry implied by the Kramer model. Using three entirely different methods, we determined the disputed ratio for the B800–850 complex of *Rh. sphaeroides* and then checked the accuracy of the methods by repeating each procedure for the B890 complex of *R. rubrum* which is generally accepted to have a ratio of 2:1.

Experimental

Wild-type cells of *R. rubrum* (strain S1) and *Rb. sphaeroides* (strain 2.4.1) were grown photosynthetically with succinate as a carbon source [19] and were harvested by centrifugation during the log phase of growth. The cells were either used immediately or stored at −20°C until required. Chromatophores of cells treated with DNAase and $MgCl_2$ were prepared by a single passage through a French pressure cell at 154 MPa [20,21]. Unbroken cells and cell wall debris were removed by centrifugation at 12000 × *g* for 15 min. The supernatant was further centrifuged at 18000 × *g* for 1 h and the chromatophore pellet was resuspended in Tris-HCl (pH 8.0) to give an A_{max} in the near-infra-red region (NIR) of 50 cm^{-1}. After solubilisation with the detergent lauryldimethylamine *N*-oxide (LDAO) (0.25% v/v), reaction centres were obtained by a low speed spin at 12000 × *g* followed by a centrifugation of the supernatant at 180000 × *g* [9]. The reaction centre-depleted pellets from the two spins were combined, resuspended in 20 mM Tris-HCl (pH 8.0) to give a NIR A_{max} of 50 cm^{-1}, and made to 1% LDAO (v/v). After a final low speed spin, antenna complexes were isolated from the pellets by DEAE-cellulose column chromatography [22]. The integrity of all antenna preparations was checked by recording their absorption spectra.

BChl-to-carotenoid ratio by HPLC. Preparation of extracts. Aliquots (100–200 µl) of antenna complex ($A_{NIR\,max}$ = approx. 30 cm^{-1}) were extracted exhaustively with approx. 5 ml acetone/methanol 7:2 (v/v) and the extracts were evaporated to dryness under nitrogen in the dark. The extracts were completely dissolved in HPLC grade dichloromethane (100–400 µl) and 20 µl samples, containing approx. 10^{-9} mol pigment, were injected immediately into the HPLC.

The HPLC equipment comprised Kontron LC-T414 pumps, Hewlett-Packard 85B interface and 1040A diode array detector. Suitable pigment separation was achieved with a Zorbax ODS (Du Pont) reversed phase column (25 × 0.46 cm) [23]. A 1 ml/min flow rate and a linear gradient of 0–100% B over 25 min was used (A = 90% acetonitrile/water, 0.5% triethylamine; B = ethyl acetate). The time taken to prepare and run each extract was approx. 45 min.

Determination of pigment absorption coefficients in eluting HPLC solvent. To calculate the molar amounts of each pigment eluted from the column it was necessary to know the absorption coefficients of BChl and the major carotenoids in the eluting HPLC solvent.

The concentration of BChl was determined from its Q_y absorption band. The millimolar absorption coefficient (ε_{590}) in acetone/methanol 7:2 (v/v) was first calculated from its peak height relative to that of the Q_y (772 nm) band, and the published ε_{772} value of 76 $mM^{-1} \cdot cm^{-1}$ [24]. This was repeated several times to give an average ε_{590} of 18.5 ± 2.0 $mM \cdot cm^{-1}$. The millimolar absorption coefficient of BChl in the eluting HPLC solvent mixture was then determined by recording the A_{max} of several samples in acetone/methanol, evaporating them to dryness, redissolving them in the same volume of eluting HPLC solvent and then by calculating the coefficient from the changes in A_{max}.

The millimolar absorption coefficients of the main carotenoids at their λ_{max} were similarly calculated in the HPLC solvent mixture from the following published $\varepsilon^{1}\%_{1cm}$ values: spirilloxanthin, 2500 in light petroleum [25]; spheroidene, 2630 and spheroidenone 2065 in acetone [26]. The

final millimolar absorption coefficients used in the ratio determinations were: BChl $\varepsilon_{580} = 17.4\ cm^{-1}$; spirilloxanthin $\varepsilon_{497} = 165\ cm^{-1}$; spheroidene $\varepsilon_{453} = 149\ cm^{-1}$ and spheroidenone $\varepsilon_{485} = 120\ cm^{-1}$.

Quantitative analysis. The diode array detector was programmed to monitor simultaneously the absorbance at the λ_{max} of each carotenoid and at the Q_x band of BChl. The absorbance area of each pigment peak in the chromatogram was automatically integrated to give the total absorbance for each pigment. The molar amounts of each pigment in the sample were then calculated using their absorption coefficients. For each ratio determination 10–17 different antenna extracts, prepared from more than one batch of cells, were used.

Before the BChl-to-carotenoid ratios were calculated, the HPLC system was calibrated for pigment losses. BChl and the major carotenoids were isolated, and spectrophotometrically determined known amounts of each pigment were injected onto the column. The molar amounts of each pigment eluted from the column, according to the HPLC analysis, were then compared to the actual amount injected and the apparent pigment losses were calculated. This was repeated 10–15 times for each major pigment.

Determination of the BChl-to-carotenoid ratios by solvent extraction. Samples of antenna complex were extracted in acetone/methanol and centrifuged at about $2000 \times g$ for 5 min. The supernatant was poured off and the extraction repeated until the pellet was colourless (usually about three times). The molar amount of BChl in the supernatant was then determined spectrophotometrically from the light absorbance of the extract at 772 nm, where there is no interference from carotenoid absorption, and the total extract was transferred into light petroleum 40–60°C b.p. The acetone/methanol mixture was removed by washing with water and the pigment-containing petroleum layer (epiphase) was dried with anhydrous sodium sulphate. The carotenoid was separated from the BChl by quantitative partitioning with 95% methanol/water [27]. The total amount of carotenoid was then determined spectrophotometrically from average absorption coefficients (*R. rubrum* $\varepsilon_{497} = 158\ mM^{-1} \cdot cm^{-1}$ and *Rb. sphaeroides* $\varepsilon_{455} = 145\ mM^{-1} \cdot cm^{-1}$) which were calculated from the carotenoid compositions (see Table II) and their individual coefficients.

Determination of the BChl-to-carotenoid ratio by difference spectroscopy. Samples of antenna complex were extracted with acetone/methanol 7:2 (v/v) as before and absorption spectra were recorded to give the molar amount of BChl in the samples. A spectrum of BChl, from an extract of the carotenoidless mutant *Rb. sphaeroides* R.26, was then recorded over the first spectrum. Increasing amounts of BChl were added to the sample cuvette until the A_{max} at 772 and 590 nm were identical to those in the extract of the antenna spectrum. Because of the presence of bacteriophaeophytin which absorbs at 772 nm it was usually impossible to match the two samples perfectly at both 590 and 772 nm but the difference was only small. The molar amount of carotenoid was then determined from the antenna extract minus BChl extract, difference spectrum, at the carotenoid λ_{max} using the average carotenoid absorption coefficients. This gave a very quick method for determining pigment ratios.

Results and Discussion

HPLC of pigments

The separation of BChl and the main carotenoids is demonstrated by the multi-channel chromatogram (Fig. 1) of a total pigment extract of *Rb. sphaeroides*. The second, smaller component of the main BChl peak was assumed to be a BChl isomer, possibly the C-10 epimer, because its absorption spectrum was identical to the main peak. It was only noted in some extracts of *Rb. sphaeroides* B800–850 complexes and cells, and when it was found the contribution of its peak area (10–15% of the main peak) was included in the ratio calculations. Bacteriophaeophytin, which is a sensitive indicator of pigment degradation, was not detected in any of the antenna complex samples. Bacteriophaeophytin, prepared from *Rb. sphaeroides* R26 BChl, was shown to have a retention time of 18.1 min.

Chromatograms of the B890 complex of *R. rubrum* and the B800–850 complex of *Rb. sphaeroides* are illustrated in Figs. 2 and 3. The average retention times (min) of each pigment were as follows: spheroidene 17.7 ± 0.5, spheroid-

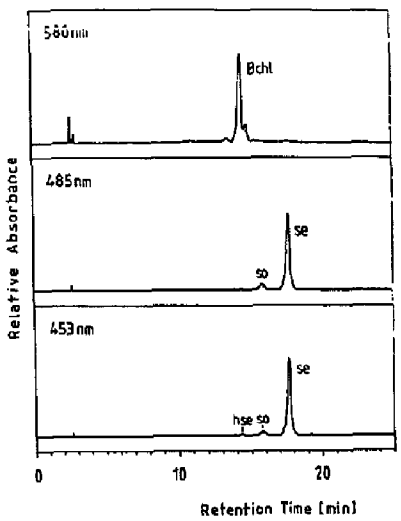

Fig. 1. 'Multi-channel' HPLC chromatogram of total pigment extract of *Rb. sphaeroides* B800–850 complex (se = spheroidene; so = spheroidenone; hse = hydroxyspheroidene). Solvent system: 0–100% B over 25 min (A = 90% acetonitrile/water, 0.5% triethylamine, B = ethyl acetate).

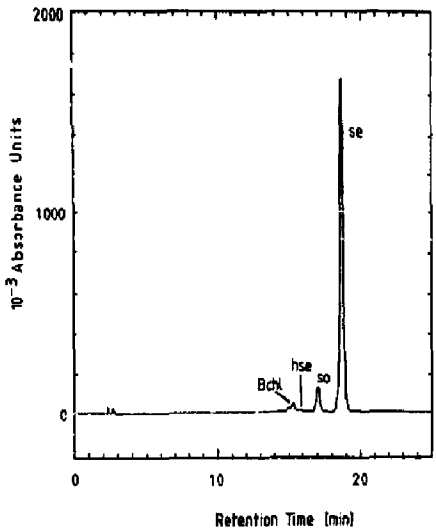

Fig. 2. Chromatogram of total pigment extract of *Rb. sphaeroides* B800–850 complex monitored at 453 nm (se = spheroidene; so = spheroidenone; hse = hydroxyspheroidene). Solvent system as Fig. 1.

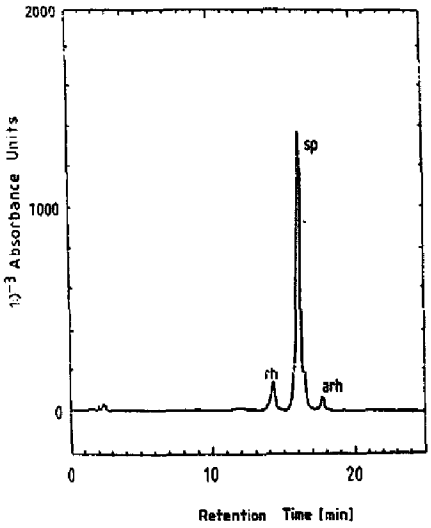

Fig. 3. Chromatogram of total pigment extract of *K. rubrum* B890 complex monitored at 497 nm (rh = rhodovibrin; sp = spirilloxanthin; arh = anhydrorhodovibrin). Solvent system as Fig. 1.

enone 16.9 ± 0.3, hydroxyspheroidene 15.8 ± 0.2, hydroxyspheroidenone 12.5 ± 0.1, rhodovibrin 14.0 ± 0.4, spirilloxanthin 15.4 ± 0.3, anhydrorhodovibrin 17.3 ± 0.3, BChl from *R. rubrum* 11.4 ± 0.2 and from *Rb. sphaeroides* 15.2 ± 0.1. The reason for the different retention times of the BChls from the two species will be discussed later.

BChl-to-carotenoid ratios

The carotenoid recoveries in extracts from both species approached 100% (Table I), as do higher plant carotenoids on similar HPLC systems [23]. Recoveries of the respective BChls of *Rb. sphaeroides* and *R. rubrum* were 64 ± 3% and 72 ± 11%. The unexpectedly high losses were proportional to sample size and were quite consistent. When corrected for these losses, the BChl-to-carotenoid ratios of total pigment extracts from *R. rubrum* B890 complex and *Rb. sphaeroides* B800–850 complex were calculated at 1.9 and 2.0, respectively (Table I). To verify these results, BChl and total carotenoid from each species were isolated and then mixed together in a known 2:1 ratio and injected into the HPLC. The ratios which were calculated directly from the HPLC

TABLE I

BACTERIOCHLOROPHYLL-CAROTENOID RATIOS

n = sample number.

	n	*R. rubrum* B890 complex ratio ± S.D.	*n*	*Rb. sphaeroides* B800–850 complex ratio ± S.D.
% recovery carotenoid	15	95 ±11	10	96 ±4
% recovery BChl	10	72 ±11	10	64 ±3
BChl/carotenoid ratio by HPLC	17	1.3± 0.2	15	1.3±0.1
BChl/carotenoid ratio corrected for losses [a]		1.9± 0.2		2.0±0.1
BChl/carotenoid ratios of 2:1 mixtures by HPLC	11	1.5± 0.3	10	1.3±0.1
BChl/carotenoid ratio by solvent extraction	9	1.7± 0.4	10	2.2±0.4
BChl/carotenoid ratio by difference spectroscopy	3	2.0± 0.3	3	2.0±0.2

[a] Assuming 100% carotenoid recovery.

data as before, were 1.5 and 1.3 for *R. rubrum* B890 complex and *Rb. sphaeroides* B800–850 complex, respectively. When corrected for BChl losses these gave BChl-to-carotenoid ratios of 2.08:1 and 2.06:1. In addition, the results obtained by the two traditional methods provided further confirmation of a 2:1 ratio for both complexes.

Carotenoid composition

HPLC analysis of antenna complex pigment extracts provided the data on their carotenoid compositions (Table II) which were used in determining the average absorption coefficients of the total carotenoid. The compositions of whole cell extracts were also determined for comparison and were found to be similar to those previously reported for *R. rubrum* (91% spirilloxanthin, 6% rhodovibrin, 2% anhydrorhodovibrin and 1% rhodopin) [28] and anaerobically grown *Rb. sphaeroides* (90% spheroidene and 10% spheroidenone) [29]. *Rb. sphaeroides* cells which have been exposed to oxygen during growth have carotenoid compositions of 95% spheroidenone, 5% neurosporene (Britton, G., unpublished results). Many other minor carotenoids are also present in cells of both species [30].

TABLE II

CAROTENOID COMPOSITION OF WHOLE CELLS AND COMPLEXES

n = sample number.

	n	Complex % ± S.D.	*n*	w/cells % ± S.D.
	B890			
R. rubrum				
spirilloxanthin	7	89.4±0.2	3	83.4±0.3
rhodovibrin	7	7.4±0.5	3	9.6±0.1
anhydrorhodovibrin	7	3.2±0.3	3	7.0±0.2
	B800–850			
Rb. sphaeroides				
spheroidene	14	93.6±1.4.	8	71 ±0.5
spheroidenone	14	4.5±1.4	8	16 ±0.1
hydroxyspheroidene	14	1.7±0.2	8	9 ±0.1
hydroxyspheroidenone	14	0	8	4 ±0.2

Bacteriochlorophyll analysis

It has been reported that the esterifying C_{20} alcohol side-chain of *Rb. sphaeroides* BChl is phytol whereas that of *R. rubrum* BChl is geranylgeraniol [31–33]. During the quantitative work it was noted that the BChls from *R. rubrum* and *Rb. sphaeroides* ran with different retention times. To investigate this further BChl samples were extracted from the carotenoidless mutants *R. rubrum* G9 and *Rb. sphaeroides* R26, mixed together, and injected into the HPLC. A two-step solvent system separated the two BChls by 4 min

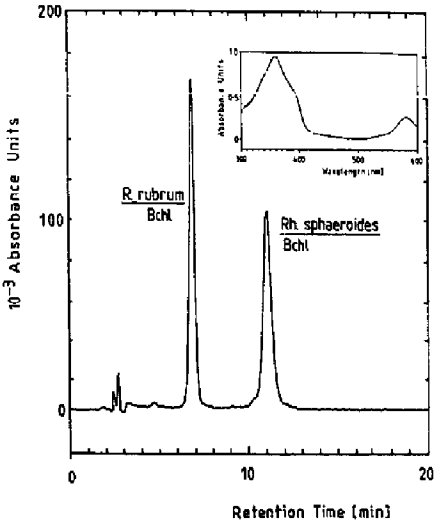

Fig. 4. Chromatogram of mixed extracts of the carotenoidless mutants *R. rubrum* G9 and *Rb. spharoides* R26, monitored at 580 nm. Solvent system: 35% B for 20 min, 35–40% B over 10 min (A = 90% acetonitrile/water, 0.5% triethylamine, B = ethyl acetate). Inset: idential absorption spectra of BChls from *R. rubrum* and *Rb. sphaeroides*.

(Fig. 4A) although the absorption spectra of the two molecules were identical (Fig. 4B). This suggests that the basis of the chromatographic separation lies in the structure of the isoprenoid side-chain and not the chromophore macrocycle and substantiates the above reports. A previous report of reversed-phase HPLC of phaeophytins esterified with different alcohols [34] has demonstrated that chlorophyll-type molecules can be separated on reversed phase HPLC on the basis of the length of the isoprenoid side chain.

In conclusion, the ratio of BChl to carotenoid in the B800–850 complex of *Rb. sphaeroides* (and probably also *Rb. capsulatus*) is 2:1 and is the same as that of the B890 complex of *R. rubrum*. these results agree with the stoichiometry used in the Kramer model.

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