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Estimation of pigment stoichiometries in photosynthetic systems of purple bacteria: special reference to the (*absence of*) second carotenoid in LH2[1]

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ABSTRACT

In this short communication we present the stoichiometric ratio of bacteriochlorophyll (Bchl), bacteriopheophytin (Bph) and carotenoids in a few photosynthetic purple bacteria complexes (whose 2D or 3D structures are well known) determined using the spectrum-reconstruction method (SRCM). An important conclusion of our pigment stoichiometric analysis is the evidence for the absence of the *second* carotenoid in LH2. In the process, we also highlight the useful application of SRCM in determining the molar extinction coefficients of carotenoids present in LH1, LH2 or RC for which these values are not known due to isolation problems and/or stability.

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[1]Abbreviations: LH2 light-harvesting complex 2, LH1 light-harvesting complex 1, RC reaction centre.

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INTRODUCTION

The pigment composition of any photosynthetic complex has traditionally been determined by procedures in which the pigments are first extracted in an organic solvent and the absorption of the mixture is then measured at a few characteristic wavelengths to determine the ratio between the pigment compositions. Recently, a more general method has been developed by Naqvi *et al.* (1). This method makes use of the individual absorption spectra of the constituent pigments in a particular solvent to reconstruct the absorption spectrum of the pigment mixture in the same solvent. Once, the experimental and reconstructed absorption spectra have been matched, the molar concentration of the pigments and/or their stoichiometric ratios can be determined using the corresponding molar extinction coefficients in that solvent. This spectrum-reconstruction method (SRCM) has already been successfully applied to analyze the chromophore composition in the light-harvesting complex (LHC II) of pea leaves (*Pisum sativum*) and to determine the total pigment content of oat leaves (*Avena sativa*) (1). We demonstrate here that this method can be used for any mixture of pigments where the absorption spectra of the constituent pigments and their molar extinction coefficients are known.

MATERIALS AND METHODS

The RCs of *Rhodobacter (Rb.) sphaeroides* 2.4.1 and R26.1 were purified according to (2). The LH1 antenna complex of *Rhodospirillum (Rs.) rubrum* S1 was extracted following the procedure described in (3) and the LH2 antenna complexes of *Rs. molischianum* and *Rhodopsuedomonas (Rps.) acidophila* 10050 were purified as in (4,5). The pigment mixture of these photosynthetic complexes were extracted in ethanol and centrifuged to pellet the unsolubilized proteins. The extracted pigment mixtures

were kept in dark until the absorption spectra were recorded. The choice of ethanol was made for several reasons. Firstly, no pigment loss is expected if the pigments in the photosynthetic complexes are directly extracted in this solvent. Secondly, the handling time is reduced and no photo-damaged products are introduced, and thirdly, if SRCM is going to be used as a standard procedure, ethanol is a common and innocuous solvent.

The individual pigments were purified using a standard procedure (6). In brief, bacteriochlorophyll *a* (Bchl-*a*) and bacteriopheophytin *a* (Bph-*a*), used in these experiments, were isolated from the LH1 complex of *Rs. rubrum* S1 and from the RC of *Rb. sphaeroides* R26.1, respectively. The carotenoids: rhodopin-glucoside, spirilloxanthin and spheroidene were isolated from LH2 of *Rps. acidophila* 10050, LH1 of *Rs. rubrum* S1 and from the whole cells of *Rb. sphaeroides* 2.4.1, respectively. Lycopene was purchased from Sigma and used without further purification. The pigments were extracted with acetone and subsequently dissolved in petroleum ether. Water was added to the mixture to introduce a phase separation. After washing several times with equal volumes of water, the pigment containing epiphase was dried with a stream of nitrogen. The pigment samples were then resuspended in petroleum ether-dichloromethane 9:1 (v/v) mixture. Subsequently, the pigments were purified by thin layer chromatography (TLC). The mobile phase was a mixture of diethyl ether and petroleum ether. The ratio (v/v) and the polar character of the mobile phase were varied according to the polarity of the pigment to be purified. The pigment spots in the TLC plates were immediately cut and soaked in ethanol. Then the extracted individual pigment solutions were filtered and kept in dark.

The absorption spectra of the pigment mixture of the photosynthetic purple bacteria complexes and of the Bchl-*a*, Bph-*a* and carotenoids were recorded in ethanol in a standard 1-cm path length cuvette using GBC 920 single beam UV/visible absorption spectrophotometer. The absorbance values were sampled at intervals of 1 nm. The baseline of each spectrum in the 800-850 nm region was corrected as suggested in (1) so as to set the average absorbance in the baseline region to zero.

The molar extinction coefficient of the Qy band of Bchl-*a* is 62000 $lmol^{-1}cm^{-1}$ in ethanol (7). The molar extinction coefficient of Qy band of Bph-*a* in ethanol was obtained by converting a known amount of Bchl-*a* to Bph-*a* by adding a single drop of concentrated hydrochloric acid into the cuvette (8,9). The calculated molar extinction coefficient was 46000 $lmol^{-1}cm^{-1}$. The molar extinction coefficient of the carotenoids used in the present study has been reported mostly in benzene but not in ethanol (10). A close inspection of carotenoids whose extinction coefficients have been determined in both ethanol and benzene (10) shows that the values in ethanol are 1.0-1.15 times of that in benzene. Thus it must be kept in mind that the molar extinction coefficients used/obtained in the present study might be underestimated by 10 to 15% as compared to the real values in ethanol.

RESULTS AND DISCUSSION

In our first experiment pigments present in the RC of *Rb. sphaeroides* R26.1 were extracted (isolated from the protein-lipid layers) in ethanol and the absorption spectrum of the free chromophores denoted - following the notation given in ref. (1) - $A_p(\lambda)$, was recorded in the 320-880 nm range. Subsequently solutions of pure Bchl-*a* and Bph-*a* were prepared in ethanol so as to give a peak absorbance between 0.5-1.0. The

absorption spectra of these solutions, denoted by Bchl(λ) and Bph(λ), were recorded. The function

$$A_m(\lambda) = \alpha Bchl(\lambda)+\beta Bph(\lambda) \qquad (1)$$

was then fitted by using a least square routine to $A_p(\lambda)$ in the 320-880 nm region[4]. From the best fit obtained the peak absorbance value of the spectra αBchl(λ) (A'_{Bchl}) and βBph(λ) (A'_{Bph}) is determined. The Bchl-*a*/Bph-*a* stoichiometric ratio can then be easily calculated using these peak absorbances and the molar extinction coefficients (ε) of the respective pigments in ethanol using equation 2:

$$C_{Bchl}/C_{Bph} = A'_{Bchl}\varepsilon_{Bph}/A'_{Bph}\varepsilon_{Bchl} \qquad (2).$$

More details of the method are found in (1). Figure 1 shows plots of $A_p(\lambda)$, $A_m(\lambda)$, αBchl(λ) and βBph(λ). For the RC of *Rb. sphaeroides* R26.1 the ratio was found to be 1.9. This ratio is in conformity with that observed in the crystal structure (11).

In a second attempt to use SRCM we undertook the pigment analysis of LH1 of *Rs. rubrum* S1. This complex contains, in addition to Bchl-*a*, spirilloxanthin as the main carotenoid (≈90%). The absorption spectrum of spirilloxanthin lies in the region of 400-550 nm region between the Soret and Qx bands of Bchl-*a* and a small contribution can be expected from Bchl-*a* in this region. The absorption spectra of Bchl-*a* and

[4] When additional pigments such as carotenoids are also involved a three parameter fitting of $A_m(\lambda)$ is performed. When Bchl-*a* and carotenoid(s) are the only absorbing chromophores, Bph-*a* is replaced by the corresponding carotenoid spectrum. Finally in

spirilloxanthin were used to reconstruct the absorption spectrum of the LH1 pigment mixture. Figure 2 shows a fairly good agreement between the experimental and reconstructed absorption spectrum of LH1. A stoichiometric Bchl-*a*/spirilloxanthin ratio of 1.95 was obtained using a molar extinction coefficient of 147200 $lmol^{-1}cm^{-1}$ for spirilloxanthin in ethanol (10). This ratio is also in very good agreement with the earlier findings in LH1 (12). In the 2D crystal of LH1 it is well established that two Bchl-*a* molecules are sandwiched in each α,β-monomer (13). This implies that one molecule of spirilloxanthin must be embedded per α,β-monomer.

We then proceed further and apply SRCM to determine the pigment stoichiometric ratio in another type of antenna complex of purple bacteria: LH2, in which there are three Bchl-*a* molecules per α,β-monomer (14,15). In this case, we decided to use LH2 from *Rs. molischianum* and *Rps. acidophila* 10050. Lycopene (>95%) and rhodopin (<5%) have been found to be the carotenoids in the LH2 of *Rs. Molischianum* (14) and rhodpin-glucoside is the one in LH2 of *Rps. acidophila* 10050 (15). These three carotenoids have 11 conjugated double bonds and can be assumed to have similar molar extinction coefficients. Figure 3 shows both the experimental and reconstructed absorption spectra of LH2 of *Rs. molischianum* in ethanol. Again a fairly good overlap of the two absorption spectra can clearly be observed. A molar extinction coefficient of 180000 $lmol^{-1}cm^{-1}$ for lycopene in ethanol was used to determine the stoichiometric ratio (10). The molar ratio of Bchl-*a*: carotenoid then turned out to be 2.9:1. Similar analysis was carried out to determine the stoichiometric ratio between Bchl-*a* and rhodopin-glucoside in LH2 of *Rps. acidophila* 10050. This also yielded 3 Bchl-*a* molecules per rhodopin-glucoside molecule. The experimental and reconstructed

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cases –see below- where the molar concentrations are known equation (2) is rewritten

absorption spectra of LH2 of *Rps. acidophila* 10050 are presented in figure 4. This ratio obtained in both the cases is higher than the values: 2:1 (14,15) or 3:2 (16) reported for LH2 of *Rs. molischianum* and *Rps. acidophila* 10050.

The results of the stoichiometric determination of all the photosynthetic systems studied are given in table 1.

Interestingly, over a period of time, a hypothesis has developed (in contrast to earlier results (17) and *references therein*) that there is a second carotenoid in LH2. Freer *et al.*, (16) find a dense region in the EM map of LH2 of *Rps. acidophila* 10050 which they interpret as either a site for the second carotenoid or that of a detergent molecule. The pigment stoichiometric ratio of *Rps. acidophila* 10050 obtained by us is not in agreement with that given in (16). The fact that the extraction of the pigments in LH2 has been carried out in the same solvent (ethanol) in which the absorption spectrum is recorded eliminates the possibility of any pigment loss in our work. This implies that there is only one carotenoid for every three Bchl-*a* molecules in the α,β-monomer of LH2 of purple bacteria. It is pertinent to mention here that in the LH2 of *Rs. molischianum* the Bchl-*a* to carotenoid ratio was also estimated to be 2:1, however a second carotenoid in the α,β-heterodimer could not be observed in the crystal structure (15).

Having successfully reproduced and determined the pigment stoichiometries in LH1, LH2 and carotenoid-free RC, we proceed further to show the application of SRCM to determine (rather approximate) the molar extinction coefficient of carotenoids in any

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accordingly to determine the unknown molar extinction coefficient.

solvent based on the known stoichiometric ratio of this carotenoid and Bchl-*a* in LH1, LH2 or RC of photosynthetic complex of any purple bacteria. As an example, we determine the molar extinction coefficient of *cis*-spheroidene in ethanol. In the RC of *Rb. sphaeroides* 2.4.1, the ratio Bchl-*a*: Bph-*a*: carotenoid (spheroidene) is known to be 4:2:1 (11,19). On the contrary, our pigment analysis gives a ratio of 4:2:0.70. The discrepancy in the molar ratio of Bchl-*a* and spheroidene is due to the fact that we have used 158000 $lmol^{-1}cm^{-1}$ as the molar extinction coefficient of spheroidene in ethanol. This value, as explained earlier, is based on the value corresponding to that of *trans*-spheroidene in benzene (10) while in the RC of *Rb. sphaeroides* 2.4.1, it is shown (18) to be most probably present in the 15-*cis*-form. The molar extinction coefficient of *cis*-spheroidene has not so far been measured in any solvent. Keeping in consideration the confidence level demonstrated so far in the accurate determination of pigment stoichiometries, we suggest that the molar extinction coefficient of *cis*-spheroidene should be around 120000 $lmol^{-1}cm^{-1}$ in order to obtain a stoichiometric ratio of 4:2:1 for Bchl-*a*: Bph-*a*: carotenoid. The 30% difference in the molar extinction coefficient of *trans*- and *cis*-spheroidene is comparable to that observed between all-*trans*- and 15-*cis*-β-carotene in hexane (19).

CONCLUSIONS

An interesting and very useful application of the spectrum-reconstruction method (SRCM) to obtain valuable information about stoichiometric pigment ratio and molar extinction coefficients of some carotenoids in some photosynthetic systems of purple bacteria has been presented. In conclusion, the two most important results of the study are: (*1*) the noticeable absence of the second carotenoid in LH2 and (*2*) the estimation of the molar extinction coefficient of *cis*-spheroidene in ethanol. Extension of this

procedure to determine the pigment stoichiometric ratio and also to estimate the molar extension coefficients of other carotenoids, especially in many-carotenoid light-harvesting complexes would substantiate the versatile application of SRCM. This is currently being attempted.

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FIGURE CAPTIONS

Figure 1:

Absorption spectrum of the pigment extract of RC of *Rb. sphaeroides* R26.1 in ethanol, $A_p(\lambda)$, given along with the reconstructed spectrum $A_m(\lambda)$. Bchl(λ) and Bph(λ) are the absorption spectra of the individual pigments in ethanol. See text for more details.

Figure 2:

Experimental (solid line) and reconstructed (solid + open-circled line) spectrum of LH1 of *Rs. rubrum S1* in ethanol. The reconstructed spectrum is a weighted-sum of the absorption spectra of Bchl-*a* and spirilloxanthin.

Figure 3:

The absorption spectrum of LH2 of *Rs. molischianum* in ethanol (solid line) plotted along with the reconstructed spectrum (solid + open-circled line).

Figure 4:

The absorption spectrum of LH2 of *Rps. acidophila* 10050 in ethanol (solid line) plotted along with the reconstructed spectrum (solid + open-circled line).

Table 1:

Stoichiometric ratios of the constituent pigments in the LH1, LH2 and RCs of purple photosynthetic bacteria determined using SRCM. The estimated error in the calculations is less than 10%.

TABLE 1

Photosynthetic system of purple bacteria	Bchl-*a* : Bph-*a*	Bph-*a* : Carotenoid	Carotenoid(s)
RC of *Rb. sphaeroides* R26.1	1.9 : 1	-	-
LH1 of *Rs. rubrum* S1	-	1.95 : 1	spirilloxanthin
LH2 of *Rs. molischianum*	-	2.9 : 1	lycopene (>95%) rhodopin (<5%)
LH2 of *Rps. acidophila* 10050	-	3.1 : 1	rhodopin-glucoside
RC of *Rb. sphaeroides* 2.4.1	2 : 1	4 : 0.70* 4 : 1*	spheroidene

* Refer to the text for more details regarding this ratio.

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Figure 1

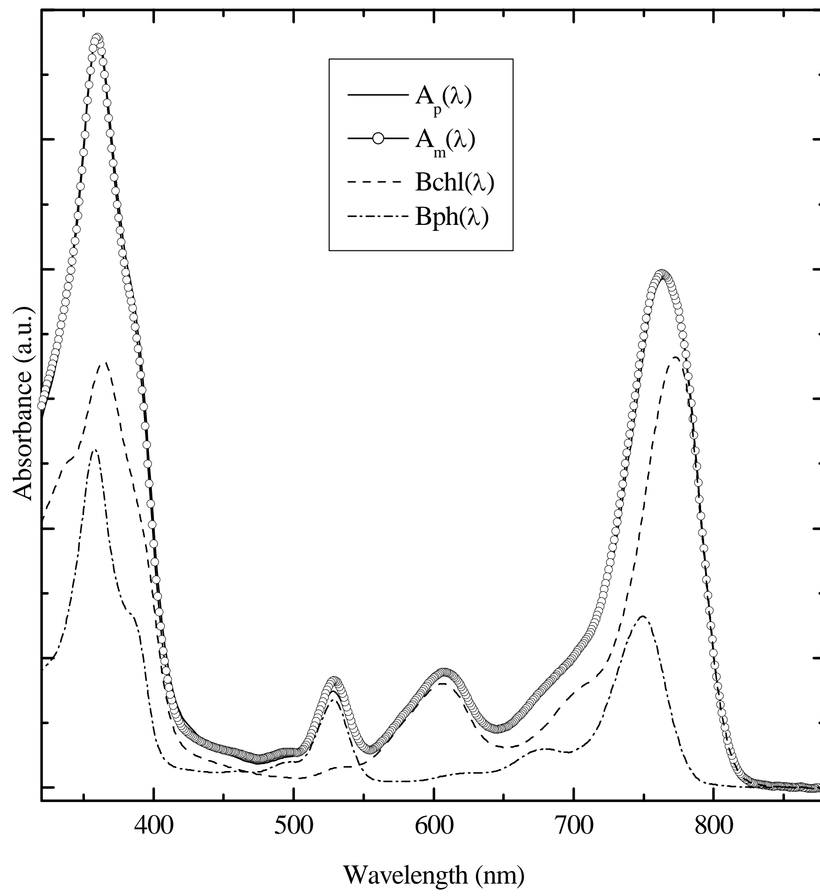

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Figure 2

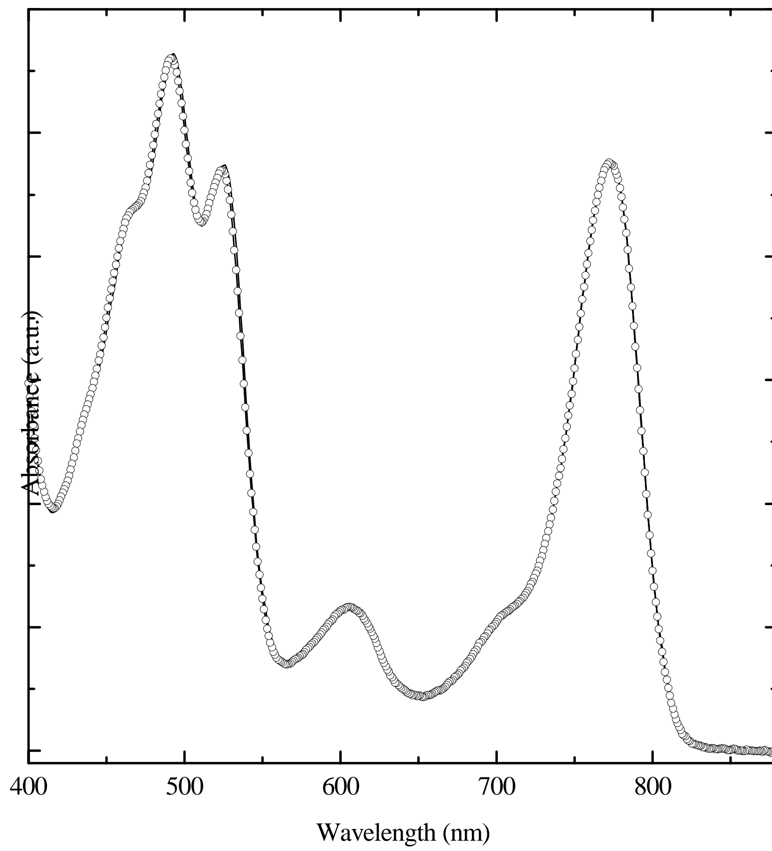

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Figure 3

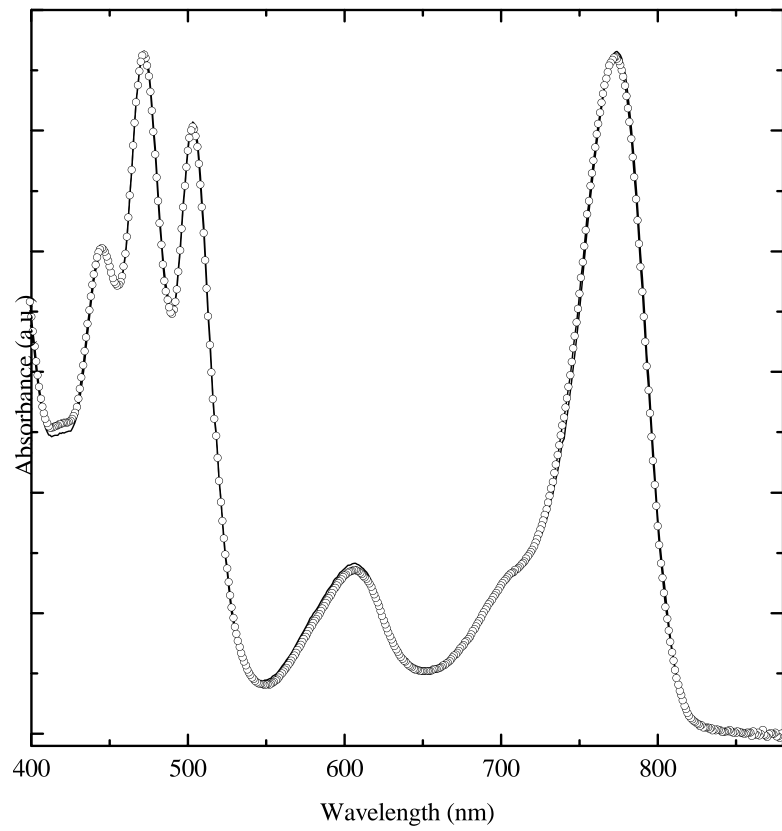

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Figure 4