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THIN-LAYER CHROMATOGRAPHY OF PIGMENTS OF THE GREEN PHOTOSYNTHETIC BACTERIUM *PROSTHECOCHLORIS AESTUARII*

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A qualitative analysis was made by thin-layer chromatography of the pigment composition of two photochemically active pigment-protein complexes from the green bacterium *Prosthecochloris aestuarii*. Both complexes were devoid of bacteriochlorophyll *c*, the most abundant pigment in intact cells, but contained bacteriochlorophyll *a*, several carotenoids, bacteriopheophytin *c* and an as yet unidentified pigment which is probably a pheophytin. The main maxima in the absorption spectrum of the latter pigment in acetone were at 411 and 665 nm; in addition, there were weak bands at 505, 535 and 605 nm. Fluorescence was emitted in a fairly broad band at 670 nm at room temperature and at 675 nm at 4 K. Two major carotenoids were detected, with absorption maxima in light petroleum at 490, 460 and 435 nm and at 503, 473 and 445 nm, respectively. The first one is probably chlorobactene or its hydroxyl derivative. The second one is probably a hydroxyl derivative of rhodopin or lycopene. In addition, at least three other carotenoids were observed in small amounts.

Introduction

Green photosynthetic bacteria posess two kinds of antenna BChl: BChl *a* and BChl *c* or *d*, the latter pigments being contained in the chlorosomes [1,2]. In addition to bands that can be attributed to BChl *a* and carotenoids, the absorption spectra of a membrane preparation and pigment-protein complexes derived from these membranes show an absorption band around 670 nm that has been attributed to BChl *c* of BPh *c* [3–5]. A strong circular dichroism signal is centered at this wavelength [5]. At low temperature the 670 nm band could be resolved into at least three different overlapping bands, belonging to pigments with different orientations with respect to the plane of the membrane [6]. At least two of these pigments showed an electrochromic response upon photooxidation of the primary electron donor P-840 [7]. These results, and the approximate constancy of the 670 nm absorption relative to the reaction center concentration, led to the conclusion that the pigments are not artifacts due to the preparation method, but that they are an intrinsic part of the intact photosynthetic system of green bacteria.

In this paper we present a study by means of thin-layer chromatography (TLC) of the pigment composition of intact cells, of the membrane preparation (Complex I), and of photochemically active pigment-protein complexes of the green photosynthetic bacterium *Prosthecochloris aestuarii*. It is shown that the absorption band around 670 nm is due to two pigments: BPh *c* and an as yet unidentified pigment resembling BPh *c*. Two major carotenoids could be detected. No evidence was obtained for the presence of BPh *a*, which seems

Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin.

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to rule out the possibility that this pigment serves as primary electron acceptor as in purple bacteria.

Material and Methods

P. aestuarii, strain 2 K, was grown as a mixed culture originally known as *Chloropseudomonas ethylica* [8] in the medium of Shaposhnikov et al. [9] containing the trace elements of Larsen [10] with versene-ol iron. The membrane preparation Complex I and the reaction center preparations, pigment-protein complex and reaction center pigment-protein complex were prepared as described elsewhere [11]. The water-soluble BChl *a* protein was prepared according to Ref. 12. Fluorescence emission and excitation spectra and low-temperature absorption spectra were measured with a single-beam spectrophotometer, equipped with a helium gas flow cryostat, as described in Refs. 13 and 14. Room-temperature absorption spectra were measured on a Cary 14 spectrophotometer.

Qualitative pigment analysis was performed on aqueous suspensions or freeze-dried samples of membrane or pigment-protein preparations, or on washed cells that were pelleted by centrifugation from a suspension in phosphate-ascorbate buffer. All experiments were done in dim light.

Samples were extracted repeatedly with acetone/methanol (7:2, v/v); sometimes an additional extraction with pure methanol was applied in order to extract all BChl *a*. After addition of light petroleum (40–60°C) the pooled extracts were washed with NaCl solution to remove acetone and methanol. The water phase was extracted again a few times with light petroleum and the pooled light petroleum extract was washed twice with water to remove salt, dried on anhydrous Na_2SO_4 and concentrated by evaporation under reduced pressure and/or in a flow of nitrogen gas. The components in this solution were separated by thin-layer chromatography on precoated silica gel plates with concentration-zone (Merck; Kiesel gel 60, 0.25 mm) developed with light petroleum (40–60°C)/acetone/2-propanol (80:10:5, v/v/v) [15]. No degradation products of BChl *a* were formed by this method, as evidenced by the fact that extraction of the antenna BChl *a* protein yielded only one blue BChl *a* spot on the chromatogram.

In some cases the samples were extracted with acetone/methanol (7:2, v/v) containing 0.1 M citric acid, a procedure that converts BChl *a* and *c* to the corresponding bacteriopheophytins [16]. The resulting solution was further treated and analyzed as described above. BPh *c* used to measure the spectra of Figs. 3 and 5 was obtained in this way by extraction of whole cells.

Results and Discussion

The results of a qualitative analysis by TLC of the pigment composition of intact cells and the various subcellular preprarations of *P. aestuarii* are shown in Table I. Except for the presence of some residual BChl *c* in Complex I, the same pigments were observed in the extracts of Complex I, the pigment-protein complex and the reaction

TABLE I

SEPARATION OF PIGMENTS BY THIN-LAYER CHROMATOGRAPHY

The separation was performed on silica gel, developed with light petroleum (40–60°C)/acetone/2-propanol (80:10:5, v/v/v). n.d., not detectable; LH, light-harvesting BChl *a* protein; (RC)PP, (reaction center) pigment-protein complex.

Spot No.	R_f	Color	Cells	LH	PP RCPP	Pigment
1	0.08	orange	+	−	+	carotenoid
2	0.16–0.22	green	+	−	−	BChl *c*
3	0.26	blue	+	+	+	BChl *a*
4	0.29	grey-green	+	−	+	BPh *c*
5	0.32	green	n.d.	−	+	unidentified (P-665)
6	0.4–0.5	yellow and pink	+	−	+	carotenoids
7	0.6	yellow	+	−	+	(hydroxy)chlorobactene

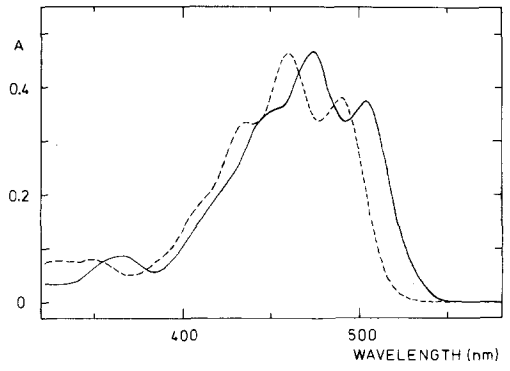

Fig. 1. Absorption spectra of the carotenoids with $R_f = 0.08$ (———) and $R_f = 0.6$ (— — —) measured in light petroleum (40–60°C). The spots were scraped off the chromatogram, extracted with acetone and subsequently transferred to petroleum ether.

center pigment-protein complex. If the acid extraction method was used, the same results were obtained, except for the conversion of BChl *a* to BPh *a*, yielding a pink spot at $R_f = 0.31$. By acid extraction of intact cells BChl *c* was also converted to its pheophytin, which gave an intense spot at $R_f = 0.27$–0.30 in the chromatogram.

The absorption spectra of the carotenoids of spot Nos. 1 and 7 are shown in Fig. 1. The carotenoid with an R_f value of about 0.6 showed bands at 490, 460 and 435 nm and is probably either chlorobactene or its hydroxyl derivative [17]. The absorption spectrum of the compound with $R_f = 0.08$ showed bands at 503, 473 and 445 nm, suggesting the presence of rhodopin or lycopene or hydroxyl derivatives of these pigments, which have approximately the same absorption spectrum [18,19]. Since rhodopin and lycopene have much higher R_f values (greater than 0.6) [17,20], this carotenoid may be a hydroxyl derivative of one of these pigments, which is retarded much more on silica gel due to its stronger hydrophilic character. If the acetone/methanol extract was saponified with methanolic KOH to remove porphyrins [19], TLC separation of the remaining carotenoid fractions yielded a chromatogram similar to that decribed above. This indicates that the low mobility of the carotenoid with $R_f = 0.08$ is not due to binding to a hydrophobic protein. The carotenoids in spots Nos. 1 and 7 were present in roughly the same amount. Liaaen-Jensen et al. [17] detected more than 90% (hydroxy)chlorobactene and less than 1% rodopin and lycopene in '*C. ethylica*', but the species involved in this case may have been *Chlorobium limicola*, rather than *P. aestuarii* (see also Ref. 21). At least three different carotenoids were present in the weak spot No. 6, which have not been further analyzed. On the basis of its absorption spectrum (Fig. 2), with maxima at 411 and 665 nm, spot No. 4 can be identified as BPh *c*, the presence of which was already suggested by Olson et al. [3].

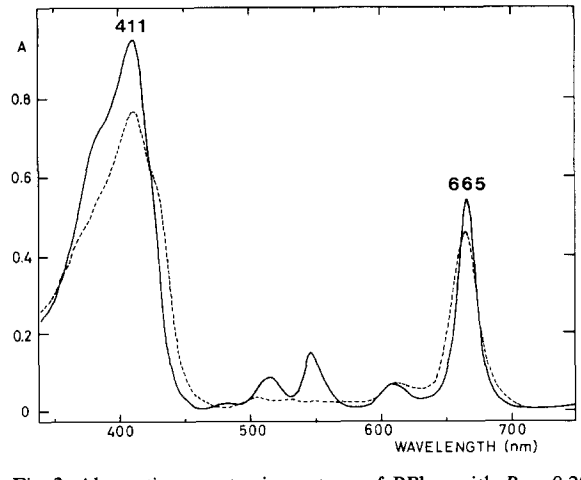

Fig. 2. Absorption spectra in acetone of BPh *c* with $R_f = 0.29$ (———) and the 'unidentified pigment P-665' with $R_f = 0.32$ (— — —).

In adddition, the chromatogram showed the presence of another pigment with a maximum at 665 nm (spot No. 5) but with an R_f value (0.32) and absorption spectrum (Fig. 2) different from those of BPh *c*. We call this pigment 'P-665'. The fluorescence emission spectra of BPh *c* and of P-665 were also different. The fluorescence spectrum of BPh *c*, measured in acetone, showed a maximum at 672 nm at room temperature and at 674 nm at 4 K; whereas the corresponding maxima for the other pigment were at 670 and at 675 nm, respectively, and, like the absorption band, the emission band was broader (Fig. 3). The fluorescence excitation spectra at room temperature and at 4 K of P-665 are shown in Fig. 4. The spectra showed the same features as the room-temperature absorption spectrum (Fig. 2), except that the bands at 505 and 535 nm were more pronounced. The main maximum in the Soret region shifted from 410 to 418 nm upon cooling, whereas the shoulder near 430 nm developed into an addi-

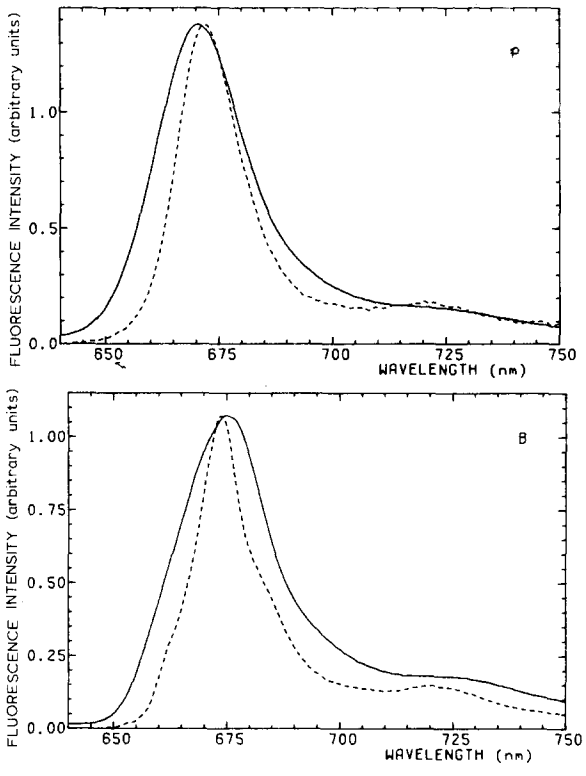

Fig. 3. Fluorescence emission spectra of BPh *c* (— — —) and of P-665 (———) in acetone. Excitation at 420 nm. (A) Room temperature; (B) 4 K. The spectra were normalized at their maxima and are plotted in arbitrary units proportional to watts per wavelength interval.

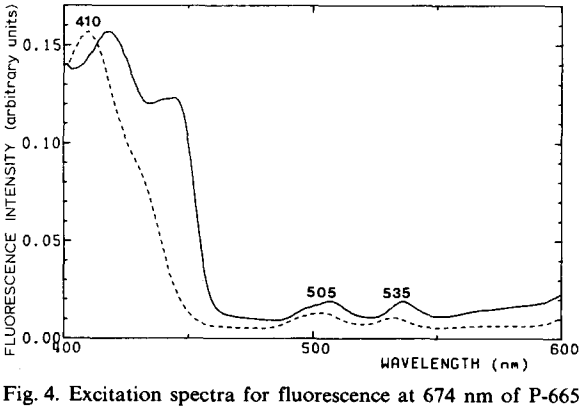

Fig. 4. Excitation spectra for fluorescence at 674 nm of P-665 dissolved in acetone, measured at room temperature (— — —) and at 4 K (———). The spectra were normalized at their maxima. The position of the maxima given in the figure apply to the room temperature spectrum.

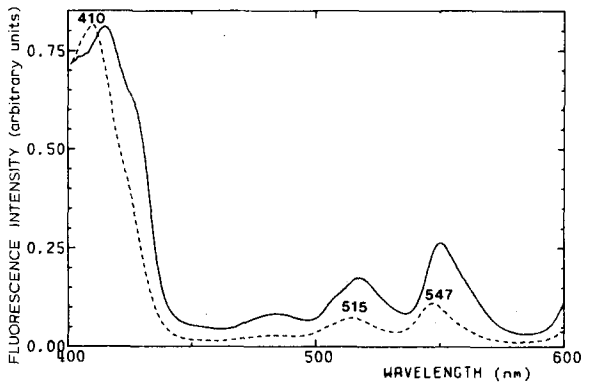

Fig. 5. Fluorescence excitation spectra of BPh *c* measured and plotted as for Fig. 4.

tional maximum near 440 nm. Fig. 5 shows the corresponding excitation spectra of BPh *c*. It should be noted that the samples were microcrystalline and highly scattering at 4 K. This may explain the apparent intensification of the bands at 484, 518 and 550 nm in the low-temperature spectrum of BPh *c*, relative to the Soret band, since the intensification due to multiple reflections in a scattering sample is more pronounced for weak than for strong absorption bands [22].

P-665 is probably a dihydroporphyrin, like chlorophyll *a* and BChl *c*. Since the acid extraction did not change its R_f value we assume that this pigment is not a chlorophyll. The presence of bands at 505 and 535 nm suggests that it is some kind of pheophytin different from BPh *c* or BPh *a*. BPh *c* and P-665 were present on the chromatograms of Complex I and the pigment-protein and reaction center pigment-protein complexes at a ratio of approx. 2:1. From this ratio and from the amplitude of the absorption band near 670 nm in the complexes [11], it can be concluded that P-665 is present at an amount of approximately two to three molecules per reaction center. BPh *c* and P-665 probably have different orientations and slightly different absorption spectra around 670 nm in vivo, as indicated by the complexity of the absorption and linear dichroism spectra at low temperature [6].

Contrary to an earlier publication [4] in which the occurrrence of a small amount of BPh *a* in a membrane preparation from *Ch. limicola* f.sp. *thiosulfatophilum* was reported, we were unable to observe this pigment in any of the extracts in the

present analysis. Taking into account the sensitivity of the analysis, this means that the concentration of BPh *a* was at least 5–10-times lower than that of P-665, which seems to rule out the possibility that BPh *a* acts as an acceptor in the light reaction, as in purple bacteria.

P-665 may be involved in energy transfer [23] and this pigment as well as BPh *c* appears to be part of the reaction center complex [23], but otherwise little can be said at present about their physiological function. It is of course tempting to speculate that P-665 may function as the intermediary electron acceptor [24] (the relatively high redox potential in vitro [25] makes such a role for BPh *c* less likely), but a test of this hypothesis will need further spectroscopic analysis of the early stages of electron transfer.

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