Tetrapyrrol Derivatives Shown by Fluorescence Emission and Excitation Spectroscopy in Cells of *Rhodopseudomonas capsulata* Adapting to Phototrophic Conditions

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Aerobically in the dark grown cells were incubated semiaerobically (30 min) and afterwards 180 min anaerobically in the light. During phototrophic induction the bacteriochlorophyll concentration increased from 0.26 to 2.10 nmol/mg cell protein.

In samples taken at different times after lowering of oxygen partial pressure the following tetrapyrrol derivatives were identified by fluorescence emission and excitation spectroscopy at 1.7 K: Mg-protoporphyrin IX-monomethylester, Mg-2,4-divinylphaeoporphyrin a_5-monomethylester, 2-devinyl-2-hydroxyethyl-chlorophyllide, 2-devinyl-2-hydroxyethyl-pheophorbide, chlorophyllide *a*, pheophorbide, bacteriopheophorbide, bacteriopheophytin, bacteriochlorophyllide and bacteriochlorophyll *a* in different pigment complexes. The highest relative concentrations of bacteriochlorophyll precursors normalized to the total amount of bacteriochlorophyll *a* were found in cells during the first hour of adaptation at 0.5 µg Bchl/mg cell protein or less.

Introduction

Bacteriochlorophyll (Bchl) and specific internal membrane polypeptides assemble in cells of phototrophic bacteria and form macromolecular complexes which have the functions of light harvesting pigments and photochemical reaction centers. The complexes are characterized by specific absorption bands in the near infrared region of the absorption spectrum, *i. e.* in *Rhodopseudomonas capsulata* at 800 and 850 nm (light-harvesting Bchl II), at 870 nm (light-harvesting Bchl I) and 800 and 880 nm (reaction center) (reviewed in [1]). It is assumed that the precursors of Bchl up to photoporphyrin IX and the peptide chains are synthesized in the cytoplasm. The last steps of Bchl synthesis (Mg-branch) and the process of assemblance of Bchl and polypeptides, however, are membrane bound processes (reviewed in [1, 2]). The possible precursors of Bchl have been identified using mutant strains blocked at different steps of Bchl biosynthesis, which enrich precursors inside the cells or excrete them into the medium [1 – 7]. The identification and localization of tetrapyrrols as possible precursors in wild type cells is difficult because of their low concentration and their lability.

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Fluorescence emission and excitation spectroscopy at low temperature has shown to be a reliable and sensitive method to detect small amounts of Bchl and Bchl light harvesting complexes as well as Bchl and chlorophyll precursors in whole cells.

During this study Bchl and tetrapyrrol derivatives were identified in whole cells taken at different times from cultures induced to synthesize the photosynthetic apparatus. The relative concentrations of the Bchl precursors have been determined.

Materials and Methods

Organism and culture. The wild type strain 37b4 of *Rhodopseudomonas capsulata* (German strain collection in Göttingen, DSM 938) was used for all experiments. The culture conditions for induction experiments have been described recently [8].

Fluorescence spectroscopy. Prompt fluorescence emission of *Rps. capsulata* was measured at 1.7 K using a spectral-fluorimeter described in [9, 10]. The excitation light source was a xenon high-pressure lamp XBO 900 W.

For measuring the fluorescence, the excitation light was passed through a water-filter, a double monochromator and appropriate cut-off-filters before being focused by quartz lenses onto the sample in the variable-temperature He-cryostat.

The fluorescence of the sample was monitored by suitable filters and a 25 cm-monochromator. The detector for the spectral range between 500 nm and 850 nm was a photomultiplier with modified S-20 cathode. Above 850 nm a photomultiplier with S-1 cathode was used. The photomultiplier current was amplified with a picoammeter and plotted with a x-t-recorder.

The *excitation spectra* in the region between 300 and 950 nm were measured at constant excitation light intensity. For this purpose part of the excitation light was diverted with a beam splitter placed behind the double monochromator and focused onto a photodiode. The current of the photodiode was amplified in a computer which generated a regulation signal for a shutter placed in the excitation path.

With this arrangement the excitation intensity could be kept constant sweeping in the whole spectral range although the intensity of the lamp varies drastically. An additional refinement of the regulation was achieved by the division of the photomultiplier current by the signal of the photodiode. These normalized fluorescence signals were plotted with a x-t-recorder.

Results and Discussion

Growth and pigment formation during induction period

Cells of *Rhodopseudomonas capsulata* grown in the dark in a medium saturated with air contained very low concentrations of bacteriochlorophyll (Bchl) and low concentrations of carotenoids (Table I). These cells had a low capacity for photophosphorylation [8, 11], not sufficient to support growth when the cells were transferred to phototrophic growth conditions (light, anaerobic).

A semiaerobic dark period reduced the lag phase of growth and induced the biosynthesis of the pigments (Table I) and of the photosynthetic apparatus [8, 12]. During the 210 min of incubation the cellular Bchl content increased 8 fold that of carotenoids only 1.4 fold. The carotenoid/Bchl ratio decreased from 6.6 to 1.2 (Table I).

The size of the photosynthetic unit increased during the induction phase due to a relative increase of light-harvesting Bchl II (B 800–850) [8]. During the 30 min of semiaerobic culture the cells do not grow. After 150 min of phototrophic induction the doubling time of protein was determined to be 10 h. The doubling time for Bchl was approximately 1 h.

Samples taken from aerobically and semiaerobically grown chemotrophic and phototrophic cultures and stopped by addition of NaN_3 and chloramphenicol were quickly frozen and studied by fluorescence spectroscopy at 1.7 K as described under Materials and Methods.

Identification of the emitting molecules

In Fig. 1 the spectra of the prompt fluorescence of *Rps. capsulata* in different stages of the phototrophic induction (Table I) are plotted. All spectra show the same emission bands differing only in their intensities and small shifts in the position of the maxima. The strong dependence of the monitored emission on excitation wavelength was utilized to identify the origins of the fluorescence bands according to [9, 10, 13]. A comparison of the excitation spectra of the maxima with those described for *Rps. sphaeroides* [9, 13] and with absorp-

Table I. Growth and pigment synthesis in cells of *Rhodopseudomonas capsulata* induced to synthesize the photosynthetic apparatus.

Incubation		cell mass OD_{660nm}	Bchl	Car	cell protein	Car/Bchl	probe
time [min]	conditions		nmol/mg cell protein		[µg/ml]	[mol/mol]	no.
0	aerobic dark	0.730	0.26	1.73	350.4	6.65	1
30	semiaerobic dark	0.730	0.27	1.79	352.4	6.63	2
90	anaerobic light	0.755	0.48	1.93	364.5	4.02	3
150		0.765	1.10	2.05	372.0	1.85	4
210		0.820	2.10	2.46	408.5	1.17	5

The culture conditions are described in Materials and Methods.
Bchl, bacteriochlorophyll; Car, carotenoids.

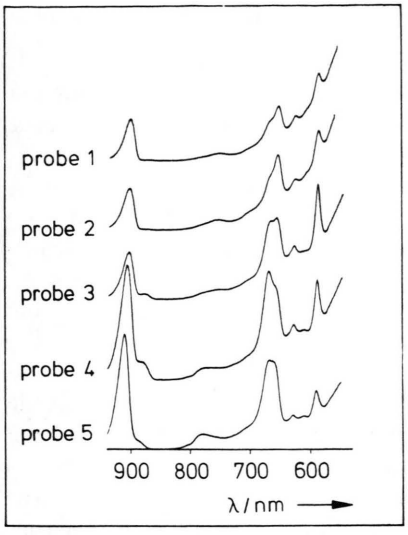

Fig. 1. Emission spectra of *Rhodopseudomonas capsulata* in different stages of growth, measured at $T = 1.7$ K with excitation at 376 nm.

tion spectra of the Bchl precursors and antenna complexes in solution allowed the identification of the participating pigments. With this method the following molecules could be attributed to the emission maxima.

Mg-Protoporphyrin IX-(mono(methyl)ester) (591 – 593 nm)

The excitation spectrum of the emission at 591–593 nm showed a strong soret band at 424 nm and a weaker maximum of 554 nm. This is exactly the same spectrum as it was determined recently [9, 10] and it allows us to attribute the emission at 591–593 nm to Mg protoporphyrin IX or its mono-(methyl)-ester.

Emission at 612,5–613 nm

The soret band of this excitation spectrum was located at 428 nm and further bands at 532 and 561 nm. Rebeiz *et al.* [14] found molecules with similar absorption and emission spectra occuring in the biosynthetic pathway of chlorophyll *a* in etioplasts. The pigments could not be identified exactly but were assumed to be intermediate stages between Mg protoporphyrin IX and Mg-2,4-divinyl-phaeoporphyrin a_5-monomethylester.

Mg-2,4-divinylphaeoporphyrin a_5-monomethylester and a precursor similar to protochlorophyll (620 and 631 nm)

The closely located broad bands could not be separated completely by excitation spectroscopy. The two soret bands were located at 419 and 446 nm and further maxima are at 538, 549, 577, 584 nm. These spectra were identical with those described by Kaiser [9, 10, 13] for the emission bands at 620 and 631 nm. It has been shown [9, 10, 13] that the excitation spectra are a superposition of the absorption spectra of Mg-2,4-divinylphaeoporphyrin a_5-monomethylester (absorption at 446, 538, 577 nm) and a pigment similar to protochlorophyll (absorption at 419, 549, 584 nm).

Mg-2-devinyl-2-hydroxyethylchlorophyllide, chlorophyllide a and pheophorbide (655–675 nm)

In the wavelength region of 655–675 nm fluorescence emission bands of several molecules were superimposed. The first component which was identified by the excitation spectra, was Mg-protoporphyrin whose vibronic bands are located at 650–658 nm. The excitation spectra of the fluorescence at 655–662 nm showed soret bands at 405 and 421 nm and weaker maxima at 505, 538, 554, 607 and 614 nm. The pigment emitting at 659 nm was identified in agreement with the results of Kaiser [9, 13] as Mg-2-devinyl-2-hydroxyethylchlorophyllide (absorption at 420 and 614 nm). The emission bands at 405, 505, 538, 554, 607 nm were found to belong to the Mg-free derivative of this chlorophyllide, which is 2-devinyl-2-hydroxyethylpheophorbide [15].

The soret bands of the emission around 672 nm were at 405, 421 and 440 nm and the other excitation maxima were detected at 510, 541, 614, 620 nm. In addition to the absorption of 2-devinyl-2-hydroxyethylchlorophyllide the excitation maxima at 440 and 620 nm indicate a molecule similar to chlorophyll *a* or chlorophyllide *a*, because there is no difference in the absorption spectra between the phytylated and unphytylated form [16]. These data and the occurrence of chlorophyllide *a* in the biosynthetic pathway to Bchl support the idea that this pigment is chlorophyllide *a*. The additional excitation bands were also a fingerprint for Mg-free derivative pheophorbide *a* [17].

The emission at 675–750 nm consisted of the vibrational bands of the pigments with fluorescence

maxima at 620, 630, 659 and 672 nm. 2-desacetyl-2α-hydroxyethylbacteriopheophorbide occuring in *Rps. sphaeroides* R 26 [10] was not detected.

Bacteriopheophorbide or bacteriopheophytin (760 nm)

The emission band at 760 nm could be seen in probe no. 3–5. The excitation maxima at 464, 538, 627, 679 nm are identical with those of bacteriopheophorbide or bacteriopheophytin. The excitation bands at 538 and 679 nm indicate a molecule in a surrounding different to the bacteriopheophytin in the reaction center.

Bacteriochlorophyllide or bacteriochlorophyll a and bacteriopheophorbide or bacteriopheophytin a (776 nm)

The excitation maxima at 362, 396, 587, 696, 711 nm are attributed to Bchl or bacteriochlorophyllide and the maxima at 362, 396, 544, 633, 670 nm to bacteriopheophytin *a* or bacteriopheophorbide in a surrounding different to the molecules emitting at 760 nm. The data of the optical spectroscopy do not allow to distinguish between the molecules with or without a phytyl chain. The phytyl chain seems to be added in the last steps of the biosynthesis [2] and this holds for the occurence of both forms in the emission spectra.

It is remarkable that the absorption bands at 696 and 711 nm of Bchl or bacteriochlorophyllide and at 663 and 670 nm of bacteriopheophytin or bacteriopheophorbide indicate two different forms for each molecule. The data support the idea that the bands at 711 and 670 nm belong to Bchl and bacteriopheophytin and those at 696 and 663 nm to bacteriochlorophyllide and bacteriopheophorbide.

LH II-complex (888 nm)

The excitation spectra at 888 nm showed bands characteristic of the LH II complex at 377, 454, 485, 514, 592, 801 and 864 nm [18–21].

LH I-complex (904–911 nm)

The maxima at 377, 456, 481, 512, 592, 802, 864, 893 nm of the excitation spectra were in agreement with the absorption spectrum of the LH I complex superimposed due to energy transfer from the LH II complex [18–21].

Quantitative evaluation, computer fit of the emission spectra

For a quantitative evaluation of the emission intensities, observed from the individual pigments, the whole emission spectrum of the probes 1–5 was fitted by a computer superimposing 11 Gaussian-lines. The only restriction was that the emission bands in the wavelength region or the precursors from 590 to 780 nm and of the Bchl-protein complexes from 870–940 nm were assumed to have the same half-width.

In addition to the 11 Gaussian-lines it was necessary to fit a background emission, derived from the fluorescence of the glycerin buffer matrix and the cell material which were also excited by the UV.

The results obtained by this procedure are explained by the evaluation of the emission spectrum of probe 5 (Fig. 2). In the upper part of Fig. 2 the emission spectra and the calculated fit and in the lower part the emission spectra and the calculated Gaussian-lines with the background were plotted.

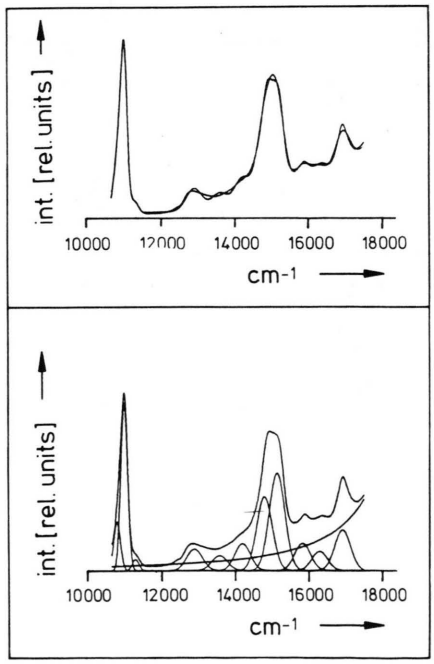

Fig. 2. Comparison of the emission spectrum (probe No. 5, $T = 1.7$ K, excitation at 376 nm) with the calculated spectrum (upper part) and with the calculated Gaussian-lines and the background (lower part).

Significance of the computer fit

The model of 11 Gaussian-lines partially coupled in their half-widths gives satisfying results. There is only an uncertainty in the background emission for which the fit was not unequivocal, leading to an absolute error in the intensities of the emission bands from 590–650 nm of about 50%. Therefore the intensities of the bands were compared relative to each other. This reduces the error to a value smaller than 20%. The tolerance in the following figures is based on the uncertainty of the background fit.

Relative concentration of the emitting molecules

The concentration of the emitting pigments in the individual stages of growth was determined by calculating the area under the Gaussian-lines and relating to the total amount of Bchl in the probes (calculated by summing up the concentrations of the bands at 870, 911 and 922 nm). In the following figures these concentrations were plotted versus the amount of µg Bchl/mg cell protein of the probes. In Fig. 3 the concentration dependence is shown for Mg protoporphyrin IX and in Fig. 4 for chlorophyllide *a*.

Bacteriochlorophyll precursors under different growth conditions

Bchl precursors were found in strong aerated chemotrophic as well as in anaerobic phototrophic cultures. A lowering of oxygen partial pressure

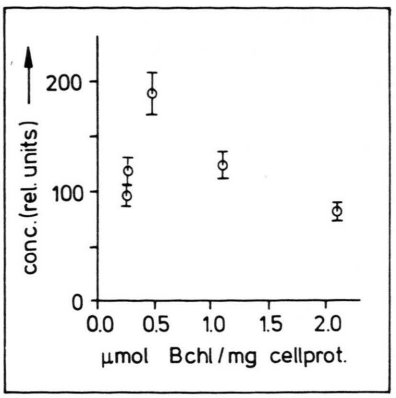

Fig. 4. Concentration of Chlorophyllide *a* (emission at 675 nm) in the different stages of growth normalized to the total amount of Bchl *a*.

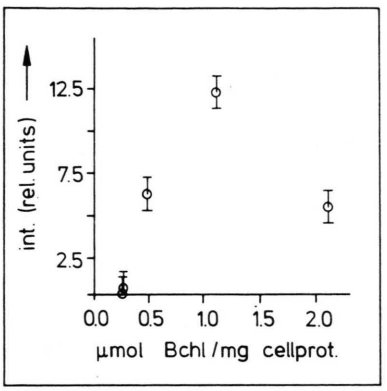

Fig. 5. Intensity of the emission of the light harvesting complex II in the different stages of growth, normalized to the total amount of Bchl.

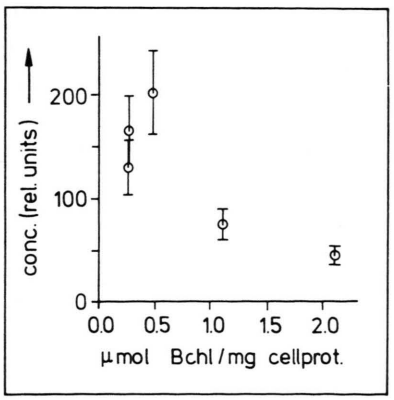

Fig. 3. Concentration of Mg protoporphyrin IX (emission at 596 nm) in the different stages of growth normalized to the total amount of Bchl *a*.

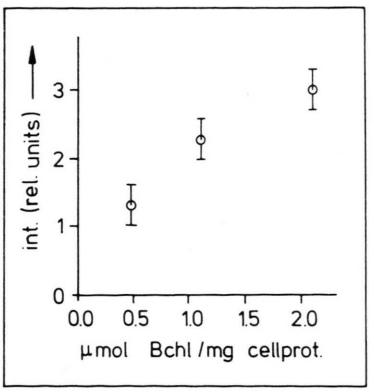

Fig.6. Intensity of the excitation band at 800 nm (LH II) in the excitation spectrum of LH I in the different stages of growth, normalized to the total amount of Bchl.

induced the formation of the photosynthetic apparatus. During induction phase an exponential or hyperexponential increase of Bchl (doubling time approx. 1 h) was observed [12]. The pool of precursors increased. As soon as the cellular Bchl concentration exceeds 0.48 nmol Bchl/mg cell protein the pool size decreased.

Light harvesting complex II

The emission intensity of the LH II complex showed a maximum at 1.1 nmol Bchl/mg cell protein (Fig. 5). The emission intensity of this complex, however, cannot be correlated to the concentration of the complex because of variations in the energy transfer between LH II and LH I Bchl in different membrane fractions during the development of the photosynthetic apparatus [8]. This assumption is supported by evaluating the excitation spectra of the emission at 911 nm. The intensity of the relative absorption maximum at 800 nm of the LH II complex, indicating the energy transfer to LH I, showed an increase when going to higher values than 1 μg Bchl/mg cell protein (Fig. 6). In contrast, the emission intensity at 800 nm decreased which could be explained by an increase in the efficiency of energy transfer rate between LH II and LH I.

Acknowledgements

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[1] G. Drews and J. Oelze, Adv. Microb. Physiol. **22,** 1 (1981).
[2] O. T. G. Jones, in: The Photosynthetic Bacteria, (R. K. Clayton, W. R. Sistrom, eds.), p. 751, Plenum press, New York 1978.
[3] J. Lascelles, in: The Photosynthetic Bacteria, (R. K. Clayton and W. R. Sistrom, eds.), p. 795, Plenum press, New York 1978.
[4] J. Lascelles, Biochem. J. **100,** 175 (1966).
[5] G. Drews, I. Leutiger, and R. Ladwig, Arch. Mikrobiol. **76,** 349 (1971).
[6] G. Drews, Arch. Microbiol. **100,** 397 (1974).
[7] J. Oelze and G. Drews, Arch. Mikrobiol. **73,** 19 (1970).
[8] A. F. Garcia, G. Drews, and H. H. Reidl, J. Bacteriol. **145,** 1121 (1981).
[9] G. H. Kaiser, Thesis, Stuttgart 1981.
[10] G. H. Kaiser, J. Beck, J. U. von Schütz, and H. C. Wolf, Biochim. Biophys. Acta **634,** 153 (1981).
[11] A. F. Garcia and G. Drews, Arch. Microbiol. **127,** 157 (1980).
[12] A. Schumacher and G. Drews, Biochim. Biophys. Acta **501,** 182 (1978).
[13] G. H. Kaiser, J. Beck, J. U. von Schütz, and H. C. Wolf, Proc. 5th Int. Congr. Photosynthesis, Halkidiki, 1980.
[14] C. A. Rebeiz, B. B. Smith, J. R. Matheis, C. C. Rebeiz, and D. F. Dayton, Arch. Biochem. Biophys. **167,** 351 (1975).
[15] W. R. Richards and J. Lascelles, Biochem. **8,** 3473 (1969).
[16] W. R. Sistrom, M. Griffiths, and R. Y. Stanier, J. Cell. Comp. Physiol. **48,** 459 (1956).
[17] J. C. Goedheer, in: The Chlorophylls, (L. P. Vernon and G. R. Seely, eds.), p. 151, Academic press, New York 1966.
[18] K. L. Zankel, Photochem. Photobiol. **10,** 259 (1969).
[19] K. L. Zankel and R. K. Clayton, Photochem. Photobiol. **9,** 7 (1969).
[20] J. C. Goedheer, Biochim. Biophys. Acta **275,** 169 (1972).
[21] R. Feick, R. van Grondelle, C. P. Rijgersberg, and G. Drews, Biochim. Biophys. Acta **593,** 241 (1980).