Degradation of Bacteriochlorophyll *a* in *Rhodopseudomonas sphaeroides* R26

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A series of pigments of the bacteriopheophytin *a* spectral type have been isolated from ageing cultures of *Rhodopseudomonas sphaeroides* strain R26. These pigments are formed in varying amounts, and can be most readily analyzed *in vivo* by their absorption in the 530 nm spectral range. They are enriched in sedimenting cells, but their formation is not affected by light. By chromatographic comparison with authentic pigments and chemical correlation, the following pigments have been identified: bacteriopheophytin *a,* bacteriopheophorbide *a* (which is the predominant product), pyrobacteriopheophorbide *a* and a fourth, very polar bacteriopheophytin *a*-type product of unknown structure. The major portion of these newly formed pigments is present in the cells in a state, in which the near-infrared absorption band is shifted to longer wavelengths. As shown by low temperature fluorescence spectroscopy, these forms are very similar to bacteriopheophorbide *a* aggregates.

Introduction

One of the most widely accepted indicators for plant senescence is the disappearance of chlorophylls. Nonetheless is the biochemistry of this process – in contrast to chlorophyll biosynthesis [1–3] and to the degradation of the structurally related hemes [4] – at present only poorly understood. In senescing green plants, products have been identified which arise from demetalation [5], hydrolysis of the phytylester [5, 6] and/or oxidation at the isocyclic ring [7, 8]. Loss of the [13^2]carbomethoxy group has been reported for *Euglena* [9]. Beyond these relatively early stages, a phytol containing pyrrol derived from ring D has been reported by Park *et al.* [10], but most of the phytol is assumed to be scavenged as fatty acid esters [11]. There is even less known about the degradation of bacterial chlorophylls.

The major handicap in chlorophyll breakdown studies is probably the lack of suitable systems, which accumulate the early products sufficiently. During a screening for suitable systems, we had observed, that cultures of the carotenoid – less mutant R26 from the purple photosynthetic bacterium, *Rhodopseudomonas spheroides,* often change color if they are kept over times extending the common growth periods of approx. 7 days. Here, we wish to report the results of a spectroscopic and chemical analysis of the major newly formed pigments, which indicate a rather similar breakdown pattern for bacteriochlorophyll *a* than has previously been reported for chlorophyll *a*.

Materials and Methods

Rp. sphaeroides (strains 2.4.1 and R26) were grown anaerobically in the light (≥ 1500 Lux from incandescent lamps) in 0.7 l bottles in Hutner's medium [12]. The cultures were not stirred. The cells were harvested by centrifugation at $10{,}000 \times g$.

Pigments were extracted with methanol according to Strain and Svec [13]. Reference pigments (see Scheme 1 for formulas and abbreviations) were prepared by the following standard procedures: Bacteriopheophytin *a*: Demetalation with dilute hydrochloric acid [14]; bacteriopheophorbide *a*: Hydrolysis with cold hydrochloric acid (18%) [15]; bacteriopyropheophytin *a*: Refluxing of bacteriopheophytin

Abbreviations: bchl, bacteriochlorophyll *a*; bphe, bacteriopheophytin *a*; bpheid, bacteriopheophorbide *a*; (see also formulas); Rp. *Rhodopseudomonas*; tlc, thin layer chromatography; HPLC, high performance liquid chromatography.

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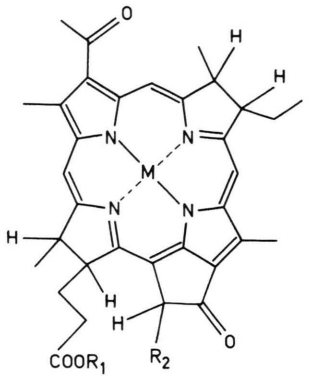


	M	R_1	R_2
bchl *a*	Mg	phytyl	$COOCH_3$
bphe *a*	2H	phytyl	$COOCH_3$
pyro-bphe *a*	2H	phytyl	H
bpheid *a*	2H	H	$COOCH_3$
pyro-bpheid *a*	2H	H	H
methyl-bpheid *a*	2H	CH_3	$COOCH_3$
pyromethyl-bpheid *a*	2H	CH_3	H

a in pyridine [16]. Methyl esters (*methyl-bpheid* and *pyromethyl-bpheid*) were prepared by refluxing in methanol containing 5% (v/v) sulfuric acid (modified from Fischer and Stern [17]). All pigment handling was done under subdued light, and all reactions were carried out under nitrogen.

Absorption spectra were recorded with a PE320 (Perkin-Elmer, Ueberlingen), a Unicam SP1800 (Philips), or a ZWS II spectrometer (Sigma, Berlin) equipped with a model 8000 "intelligent recorder" (Bryans, Mitcham).

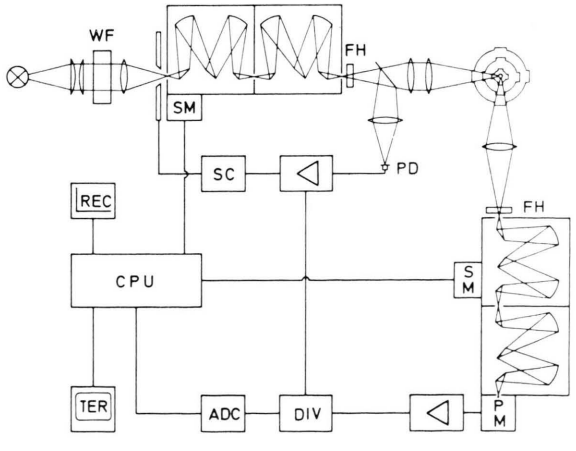


Fig. 1. Apparatus for fluorescence measurements. See materials and methods for details.

Fluorescence spectra were recorded with a home-built apparatus (Fig. 1). The light of a Xenon arc lamp (Osram XBO 900) was focused on the entrance slit of a double monochromator (Spex 1672). Part of the light leaving the monochromator was reflected by a quartz plate onto a photodiode (PD). The signal of the photodiode was used as a reference for the slit control (SC). It controled a variable slit at the entrance of the monochromator to obtain a constant excitation intensity over the entire spectral range. The sample was cooled to 4K in a liquid helium cryostate. The light emited by the sample passed a double monochromator (Spex 1680) and was detected by a photomultiplier (PM) (S1 photocathode, type EMI 9684 for near infrared detection; S20 photocathode, type EMI 9558 for the visible region). In order to compensate for the intensity minima in the spectral output of the xenon lamp, which cannot be adequately regulated by the slit control, the amplified signal from the photomultiplier was divided by the signal from the photodiode.

Analytical thin layer chromatography was done on reverse phase HPTLC plates (Merck, Darmstadt, RP-8, F254S) and methanol or mixtures of methanol/water (95:5, v/v) as eluents. HPLC was performed on a system consisting of 2 model 6000A pumps and a model 660A solvent programer (Waters, Königstein), a LiChrosorb RP-18 column 250×4.6 mm (Merck, Darmstadt) and two absorption detectors model spectro monitor II (Latek, Heidelberg) set at 525 and 595 nm. Flow: 1.5 ml/min, solvent A: methanol–water = 80:20 (v/v), solvent B: ethyl acetate, program #8 from 0 to 50% B in 30 min.

Results

In vivo absorption spectra

Ageing cultures of *Rp. spheroides* R26 change gradually their color from a clear blueish-green to a violet hue. The time evolution of this color change is rather different for different cultures and culture conditions. Two extreme situations are illustrated in Fig. 2. The slowly degrading culture (Fig. 2a) shows over a period of 46 days a decrease of the bchl Qx-band at 592 nm by about 50%. At the same time, there is an increase of a broad and structured band around 530 nm. This region is typical for the Qx-band of demetalated bchl, *e.g.* bacteriopheophytin (bphe) type pigments. The changes in the Qy region

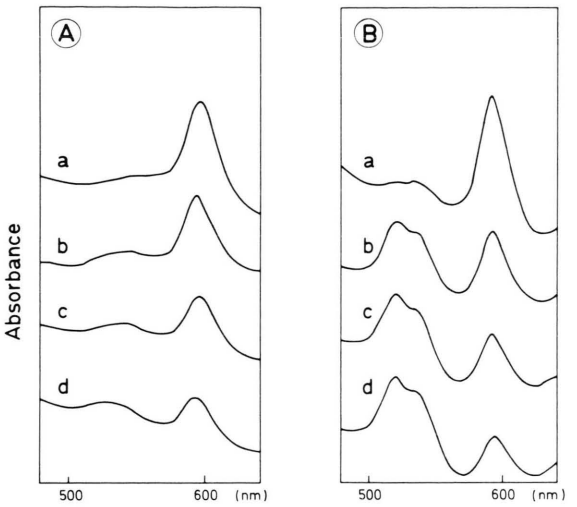


Fig. 2. *In vivo* absorption spectra (Qx region of the tetrapyrrolic pigments) of ageing cultures of *Rhodopseudomonas sphaeroides* R26. A: Slowly degrading culture after 6 (a), 14 (b), 22 (c) and 46 days (d) of inoculation; B: Rapidly degrading culture after 4 (a), 14 (b), 20 (c) and 27 days.

are less significant with a uniform decrease and a minor blue-shift (see Fig. 3).

The fast degrading culture shows qualitatively the same changes, but both the decrease around 590 nm and in particular the increase around 530 nm are much more pronounced and rapid (Fig. 2b). In this culture, the 530 nm region shows two well resolved maxima at 522 and 538 nm. The difference between the two cultures is as yet only poorly defined. It is noteworthy, however, that light is not an important factor. Parallel cultures grown under otherwise identical conditions in the dark or under continuous illumination ($\leq$ 2000 lux), respectively, yield the same spectra.

The occurrence of a prominent absorption around 530 nm is accompanied by the formation of a purplish sediment, which in light grown cultures may also extend to the walls of the culture vessel facing the light source, where generally a blanket of cells develops in cultures which are more than 10 days old. The sediments show the aforementioned spectral changes in the Qx band region much more clearly than the still suspended cells. Pronounced changes are visible even in the sediments of slowly degrading cultures. Moreover, the sedimented cells show also distinct changes in the Qy region which was obscured in the absorption spectra of the whole culture (Fig. 3, trace c). The maximum of this band is shifted to 860 nm, and second band arises at its short-wavelength shoulder (λ_{max} = 805 nm). In the fast degrading culture, the sediment is completely free of bchl if judged from the absence of the Qx band around 600 nm, and the 805 nm band becomes a distinct absorption band in the near infrared region (data not shown).

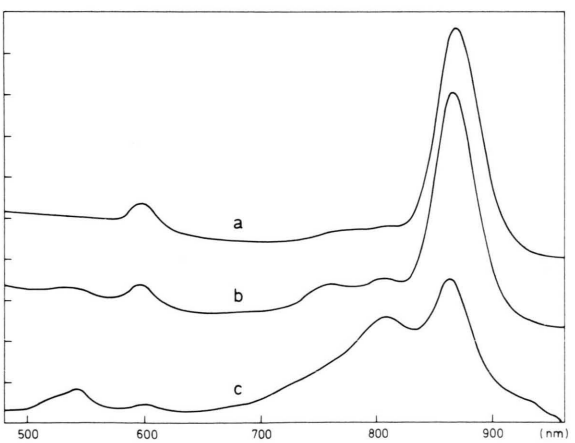


Fig. 3. *In vivo* absorption spectra of a slowly degrading culture of *Rhodopseudomonas sphaeroides* R26 after 6 (a) and 46 days (b) of inoculation, and sedimented cells after 30 days of inoculation (c).

Identification of the pigments

The spectra of the methanolic extracts of aged cultures are typical for pigment mixtures of the bchl and bphe spectral type, which can be analyzed most readily by their Qx absorptions around 600 and 530 nm, respectively. In contrast to the *in vivo* spectra, the extracts have only a single, unstructured absorption around 530 nm (data not shown). Tlc and HPLC analysis of the extracts yielded besides bchl several fractions which all have the same absorption spectrum identical to bphe. The following pigments have been found in the extracts: Bacteriopheophytin *a* (bphe), bacteriopheophorbide *a* (bpheid), bacteriopyropheophorbide *a* (pyro-bpheid) and a fourth, unidentified pigment. The latter is the most polar product in the extract and is only found in the sediment of fast degrading cultures. The identification of these pigments is based on (a) their absorption spectra, (b) chromatographic comparison (tlc, HPLC) with authentic samples (s. Table I) and (c) conversion to the methyl esters and subsequent

Pigment	bchl *a*	bphe *a*	pyro-bphe *a*	bpheid *a*	pyro-bpheid *a*
Retention time [min]	32	34	36	6	9

Table I. Retention times of bacteriochlorophyll *a* and its degradation products (see materials and methods for details of the HPLC system).

chromatographic comparison with the authentic methyl- and pyromethyl-bacteriopheophorbides *a*. The quantitative analysis of a fast degrading culture at different times, as well as of the sediments after prolonged standing, is given in Table II. The bchl content decreases continuously in all cultures, and the major new products are the unesterified, demetalated pigments, *e.g.* bpheid and pyro-bpheid. The relative amounts of these pigments varies, but bpheid is generally formed in larger amounts than is pyro-bpheid (the latter is absent in slowly degrading cultures). The esterified, demetalated pigment, bphe, never amounts to more than 5% of the total pigment.

Fluorescence spectra

Fresh cultures of *Rp. spheroides* R 26 have a single major emission maximum at about 910 nm, with a typical bchl excitation profile [18]. Other pigments have been reported to be present in minor amounts, most likely as biosynthetic precursors of bchl. A slowly degrading culture of this bacterium shows essentially the same major fluorescence pattern (Fig. 4a, excitation at 600 or 400 nm, Fig. 4b, emission at 925 nm). However, a distinct second near-infrared emission maximum is visible at 873 nm under conditions favorable for the excitation of bphe type pigments (Fig. 4a, excitation at 525 and 400 nm). This assignment is supported by the excitation spectra with typical maxima in the 530 nm region and the absence of a 600 nm maximum (Fig. 4b, emission at 865 nm). Interestingly, two types of bphe-like pigments can be resolved by fluorescence spectroscopy. The major one emitting around 860 nm has a split and relatively weak Qx band, whereas a minor component emitting at about 770 nm has a more intense and unstructured Qx band.

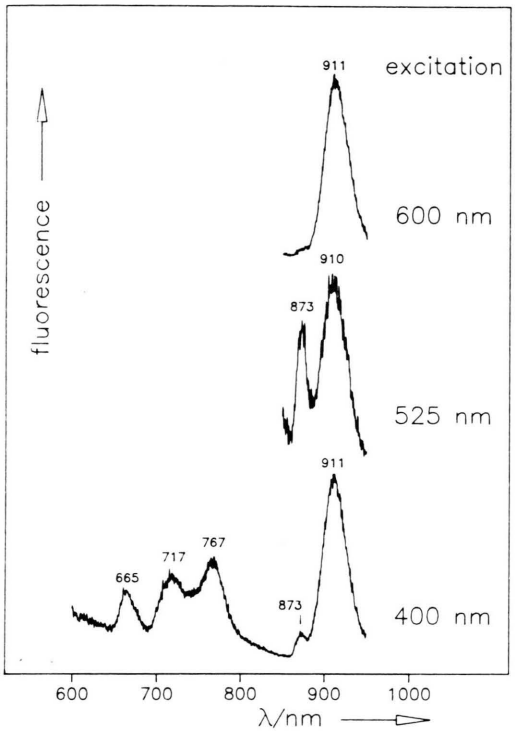
These changes are again most pronounced in the sediment of fast degrading cultures. Only traces of bchl can be detected in the excitation spectra (Fig. 5b, emission at 909 nm), and the two distinct spectral forms of bphe-type pigments are clearly visible both in the emission (Fig. 5a) and the excitation spectra (Fig. 5b). In the sediments, both forms are present at about the same amounts.

The shorter wavelength (754 nm) emitting form has spectral properties which are typical for bphe-type pigments in monomeric solution. Excitation and emission spectra of bpheid are shown in Fig. 6 for comparison. The excitation spectra of the longer wavelength form are very similar to the absorption of bphe aggregates in micellar or mixed aqueous/organic solvents [19, 20].

Since no data were available on the aggregation of the respective unesterified pigment, such studies have been performed with bpheid. The absorption spectra indicate a similar, although less pronounced aggregation both in detergent micelles and in aqueous glycerol. The low temperature fluorescence spectra of bpheid in the latter system are shown in Fig. 7. Monomeric and aggregated bpheid can be clearly distinguished by their excitation and emission spectra. The former emitting at 775 nm has a single and relatively intense Qx excitation band at 539 nm (Fig. 7a, emission at 780 nm), which is only slightly shifted as compared to the methanol/glycerol solution (Fig. 6). The aggregates emitting at 900 nm have a split and decreased Qx excitation band (λ_{max} = 510 and 528 nm).

Table II. Pigment composition in ageing cultures of *Rhodopseudomonas sphaeroides* R 26. The data are taken from the HPLC analysis of the samples shown in Fig. 1B, all values are given in (%) of the total tetrapyrroles (bacteriochlorophyll + bacteriopheophytin type pigments). "X" is the unknown polar compound with bphe type absorption spectrum (see text). The same extinction coefficients have been assumed for all the pheophytin-type pigments in the analysis. See Fig. 2 for the absolute decrease in pigment contents, which is always ≤ 50% but difficult to quantify from the *in vivo* spectra due to the uncertainties in the extinction coefficients.

Age of culture [days]	bchl *a*	bphe *a*	bpheid *a*	pyro-bpheid *a*	X
4	90	2	2	6	–
14	56	4	31	5	4
60	–	–	46	10	44



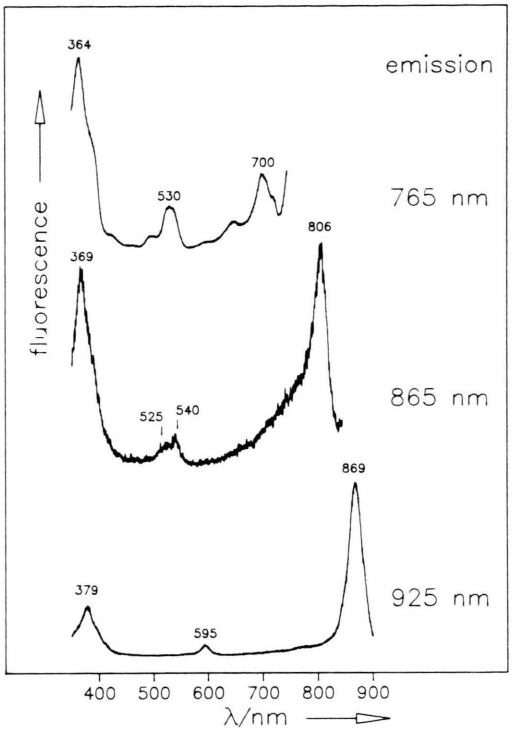
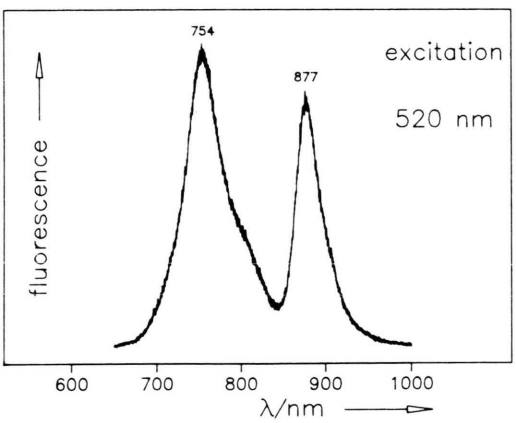


Fig. 4. Low temperature (4K) fluorescence emission (A, left) and excitation spectra (B, right) of a 28 day old slowly degrading culture of *Rhodopseudomonas sphaeroides*.



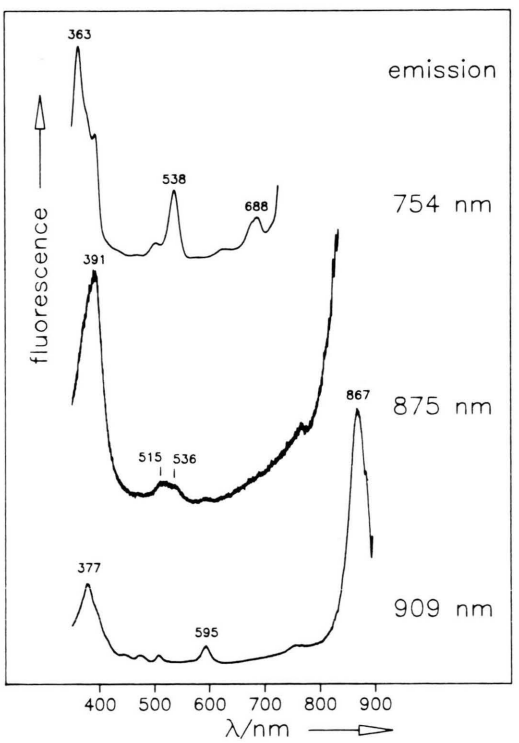


Fig. 5. Low temperature (4K) fluorescence emission (A, left) and excitation spectra (B, right) of the sedimented cells of a fast degrading culture.

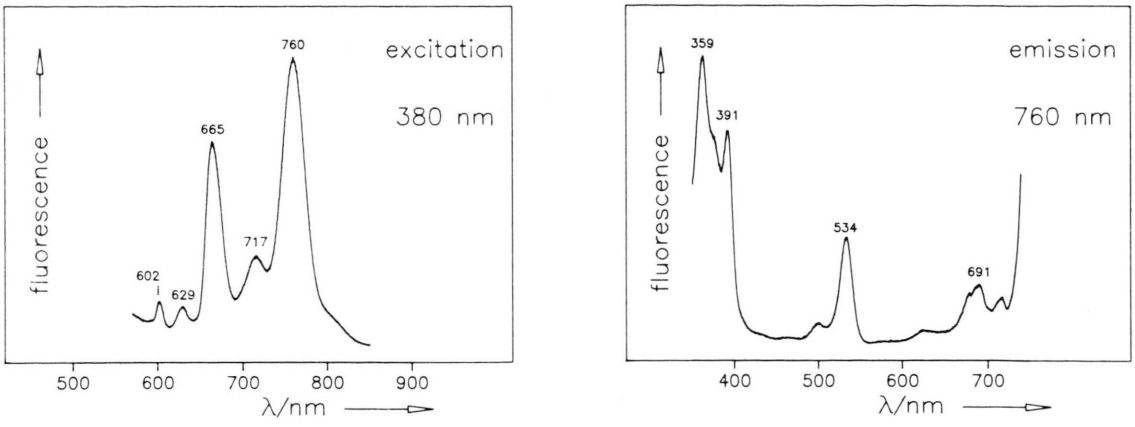


Fig. 6. Low temperature (4K) fluorescence emission (A, left) and excitation spectra (B, right) of monomeric bacteriopheophorbide *a* in methanol/glycerol = 1:1. The emission band at 665 nm in (A) and the excitation band at 691 nm (B) are due to impurities.

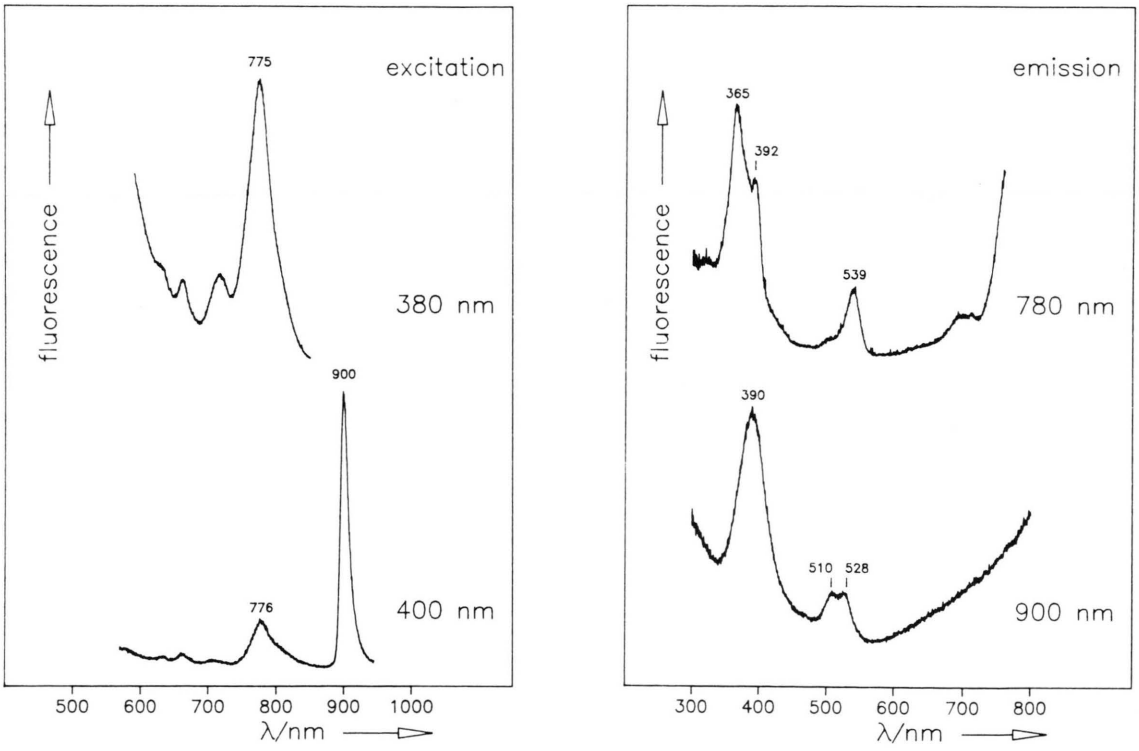


Fig. 7. Low temperature (4K) fluorescence emission (A, left) and excitation spectra (B, right) of aggregated bacteriopheophorbide *a* in water/glycerol = 1:1.

Discussion

All the degradation products investigated are of the bacteriopheophytin *a* spectral type. The degradation is thus most easily detectable in the 530 nm spectral range, where bacteriopheophytins but not the related bacteriochlorophylls possess a moderately intense absorption band. This finding is in agreement with the reports on degradation of chlorophyll *a* in higher plants, where the demetalation to pheophytin

a-type pigments seems to be an early or even the first degradation step [5, 9, 21, 22]. There are furthermore distinct similarities in the nature of structural modifications among the pheophytin-type pigments. Products arising from the hydrolysis of the propionic acid phytyl ester as well as from the decarbomethoxylation at C-13^2 are found for both the plant and bacterial chlorophylls. However, no 13^2-hydroxy bacteriochlorophylls have been observed under our experimental conditions, although bacteriochlorophyll and its pheophytin are amenable to ready oxidation at this position during extraction [23, 24].

The changes in the *in vivo* absorption spectra are most significant in the spectral region around 530 nm. This absorption originates from the Qx transition of the demetalated bacteriochlorophyll type pigments, *e.g.* bacteriopheophytin and its derivatives. Since there is no corresponding increase in the spectral region around 750 nm related to the Qy band of these pigments, the major portion must be present in a red-shifted spectral form. Long wavelength absorbing forms of bacteriochlorophylls are characteristic of most of the functional bacteriochlorophyll-protein complexes of the photosynthetic apparatus. Long-wavelength absorbing forms of bacteriochlorophylls *and* bacteriopheophytins are formed, too, with many natural and synthetic amphiphiles and also in mixed aqueous-organic solvents (see [19, 20, 25–29]). In both cases, they have been related to aggregation of the pigments. Unlike the well characterized *in vitro*-aggregates which are typical for the green chlorophylls [30, 31], this aggregation is in the bacteriochlorophyll series not dependent on the presence of the central Mg atom [19, 20].

All earlier studies have been performed with esterified pigments. However, the propionic acid side chain is not esterified in the major portion of the degradation products identified in ageing *Rp. sphaeroides*. We have, therefore, studied the aggregation of bacteriopheophorbide *a*. The absorption (not shown) and in particular the low temperature fluorescence spectra show clearly that the aggregation is principally similar with the esterified pigments. The question then arises whether the pigments in the ageing cells are still bound to proteins, or possibly present as aggregates similar to the ones observed *in vitro*. A detailed inspection of the fluorescence spectra of whole cells revealed small but distinct shifts as compared to the aggregates formed in aqueous glycerol. While these results clearly show a different environment, further studies are required to identify the state of the pigments within the cells.

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