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The nature of the primary electron acceptor in green sulfur bacteria

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It was shown previously (Van de Meent, E.J., Kobayashi, M., Erkelens, C., Van Veelen, P.A., Amesz, J. and Watanabe, T. (1991) Biochim. Biophys. Acta 1058, 356–362) by means of HPLC, NMR and optical and mass spectroscopy that the primary electron acceptor of heliobacteria is 8[1]-hydroxychlorophyll (Chl) *a*. In view of the spectral and functional similarities between this pigment and the primary electron acceptor of green sulfur bacteria, we have applied the same methods to various species of green sulfur bacteria (*Prosthecochloris aestuarii, Chlorobium limicola, C. limicola f. thiosulfatophilum, C. vibrioforme* and *C. phaeovibrioides*) in order to study the identity and the occurrence of the latter pigment. It was already shown from flash spectroscopic and reversed phase HPLC experiments on isolated membranes and solubilized membrane fractions of *P. aestuarii* that the most likely candidate for the primary acceptor is a pigment named bacteriochlorophyll (BChl) 663, which had been tentatively identified as a lipophilic form of BChl *c*. In this communication we will show by means of optical spectroscopy, ^{252}Cf-plasma desorption mass spectroscopy and ^{1}H-NMR that BChl 663 is an isomer of Chl *a*. This result again emphasizes the similarities between the reaction centers of green sulfur bacteria, heliobacteria and Photosystem I. By means of normal-phase HPLC analysis of the five species of green sulfur bacteria it is shown that BChl 663 is universally present and in comparable quantities in this group of photosynthetic bacteria. No other pigments with similar spectroscopic properties were detected.

Introduction

Photosynthetic green sulfur bacteria have an extensive antenna system which shows a considerable complexity [1]. The bulk of the pigments are contained in the so-called chlorosomes, oblong bodies of several tenths of nanometers diameter, attached to the cytoplasmic membrane. Depending on the species, they contain mainly bacteriochlorophyll (BChl) *c*, *d* or *e*. In addition to this, chlorosomes contain carotenoids and small amounts of BChl *a* [2–5]. Most of the BChl *a*, present in a 10–20-times lower amount than BChl *c*, *d* or *e*, is located in the cytoplasmic membrane and in a water-soluble BChl *a* protein complex [6–9]. The latter is attached to the membrane and is thought to be situated between the chlorosome and the membrane [6,10]. The reaction center of green sulfur bacteria appears to be functionally and structurally different from that of purple bacteria, and more related to that of heliobacteria and Photosystem I (PS I) [1,11].

Isolated membranes of green sulfur bacteria [8,9], as well as solubilized fractions derived from these membranes [12–14], show a conspicuous absorption band near 670 nm, which is associated with the so-called core complex. This complex also contains the reaction center [14]. Reversed-phase HPLC on isolated and solubilized membranes from *Prosthecochloris aestuarii* resulted in the isolation of BChl 663, which was tentatively identified as a lipophilic form of BChl *c* [15]. Its presence was also observed in intact cells of *Chlorobium limicola* [16], which shows that BChl 663 is not an artifact produced during membrane isolation. Flash-

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Abbreviations: RC, reaction center; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; CD, circular dichroism; Chl, chlorophyll; COSY, correlated spectroscopy; HPLC, high-performance liquid chromatography; *P*-840, primary electron donor; Phe, pheophytin.

spectroscopic evidence showed that at least part of the BChl 663 present is associated with the reaction center and functions as the primary electron acceptor [17–19].

By means of normal-phase HPLC, NMR and ^{252}Cf-plasma desorption mass-spectrometry (^{252}Cf-PDMS) we have shown that the primary electron acceptor of heliobacteria is 8^1-hydroxy Chl *a* [20]. In view of the spectral and functional similarities between this pigment and BChl 663, we applied the same methods to various species of green sulfur bacteria in order to study the identity of the latter pigment. It will be shown by means of optical spectroscopy, ^{252}Cf-PDMS and ^{1}H-NMR that BChl 663 is an isomer of Chl *a*, which once again emphasizes the similarities between green sulfur bacteria, heliobacteria and PS I. Evidence will be given, based on normal-phase HPLC analysis of five species of green sulfur bacteria, *P. aestuarii*, *C. limicola*, *C. limicola f. thiosulfatophilum*, *C. vibrioforme* and *C. phaeovibrioides*, that the structure of BChl 663 is identical for all species of green sulfur bacteria.

Materials and Methods

Culturing and sample preparation

Prosthecochloris aestuarii was cultured while stirring under anaerobic conditions in a mixed culture originally known as *'Chloropseudomonas ethylica'* [21] in the medium described in Ref. 22 at 1000 lux illumination from incandescent lamps in 12 l batches at an ambient temperature of 30°C. In order to obtain sufficient material for pigment isolation and analysis a total of 120 l *P. aestuarii* culture was processed.

Chlorobium vibrioforme (strain 6030) and *C. phaeovibrioides* (strain 2631) were a kind gift by Dr. N. Pfennig (Konstanz). They were cultured as described in Ref. 23 under 500 lux illumination in 1-liter batches at an ambient temperature of 30°C. *C. limicola* and *C. limicola f. thiosulphatophilum* were grown as described in Ref. 24.

Cells were harvested by centrifugation at 12000 × *g*. Subsequently, the cells were washed with 10 mM phosphate buffer (pH 7.4) containing 10 mM ascorbate. The washed cells were resuspended in the same buffer and sonicated for 45 min in the dark, cooled in an ice/water bath. Remaining whole cells were pelleted by centrifugation. The resulting sonicated cell preparation was used for further study.

Pigment isolation

Pigment extraction was performed as described earlier [25]. In short, the extraction was accomplished by mild sonication of sonicated cells in a approx. 50-fold volume of acetone during 2 min at 4°C in the dark. The extract was filtered and subsequently dried on a rotary evaporator. This procedure was completed within 5 min. Repeating the extraction procedure on the residual of a sample of *P. aestuarii* (obtained by centrifugation) showed an extraction efficiency of higher than 99% on a BChl *c* basis.

The brownish-green solid material obtained by the extraction procedure was dissolved in chloroform. Approx. 3 µl of the solution was injected into a silica HPLC column (Senshupak 1251-N, 250 × 4.6 mm i.d.) cooled to 4°C in an ice/water bath. The pigments were eluted isocratically with degassed n-hexane/2-propanol/methanol (100 : 1.0 : 0.15, v/v) at a flow rate of 1 ml min^{-1}. Pigment elution was monitored by means of an Applied Biosystems Spectroflow 757 UV/VIS-detector. The signal was digitized using a home-built analog-to-digital converter-amplifier unit and stored on a personal computer for later analysis. In some cases a JASCO Multiwavelength 340 detector was used to monitor the elution pattern and the absorption spectra of the eluting components simultaneously.

Sufficiently pure pigments for the measurement of absorption, mass and ^{1}H-NMR spectra were prepared by means of preparative-scale HPLC (Senshupak 5251-N, 250 × 20 mm i.d.) with n-hexane/2-propanol/methanol (100 : 1.5–2.3 : 0.15, v/v) at a flow rate of 5 ml min^{-1}. The solvents used for analytical and preparative HPLC were analytical and reagent grade, respectively, and kept under a nitrogen atmosphere after degassing. Pure Chl *a* was obtained by preparative scale chromatography of extracted membranes of *Anabaena cylindrica* or spinach. Acid treatment of Chl *a* in chloroform was used to yield Phe *a*, which was subsequently purified. Epimerization of Phe *a* was brought about by overnight storage of a thoroughly degassed acetone solution under low pressure at room temperature.

Spectroscopy

Absorption and circular dichroism spectra were measured as described earlier [25].

^{1}H-NMR spectral measurements were performed on a Bruker WM-300 spectrometer. All NMR experiments were conducted in [$^2H_6$]acetone (Janssen Chimica, Belgium), at 243 K to reduce degradation reactions and to enhance spectral resolution. Shortly before the measurement all solutions were repeatedly frozen and degassed at 10^{-3} Torr, after which the tube was sealed. ^{252}Cf-plasma desorption mass spectrometry was performed by means of a Bio-Ion 20 spectrometer from Applied Biosystems, Uppsala, Sweden. The pigment was dissolved in acetone and transferred onto a nitrocellulose target where the solvent was evaporated.

Results and Discussion

Chromatographic analysis

Fig. 1 shows an elution pattern obtained by normal-phase HPLC of an extract of sonicated cells of *P.*

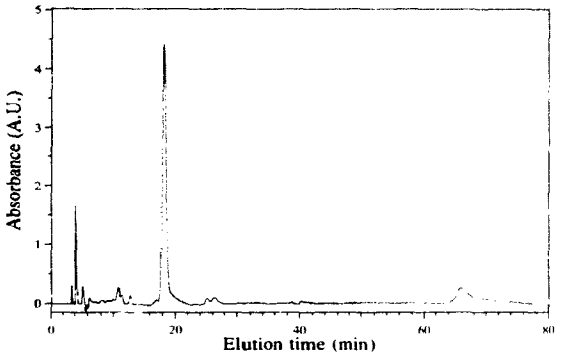

Fig. 1. Chromatogram of *P. aestuarii*, monitored at 663 nm. A.U., arbitrary units.

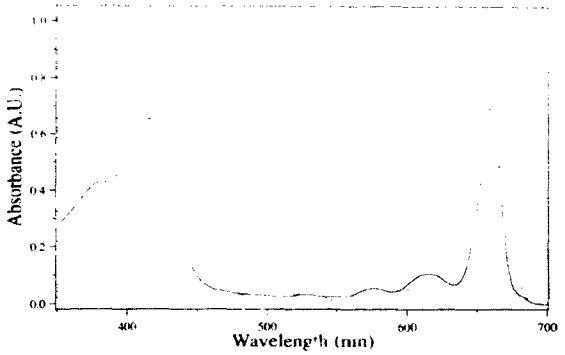

Fig 2. Absorption spectra in diethylether of BChl 663 (solid line) and its pheophytin, BPhe 663 (broken line).

aestuarii. The absorption of the eluent was monitored at 663 nm, where BChl *a* and carotenoids show no or only negligible absorption.

The most prominent peak eluted near 19 min. This peak was also present in the elution diagrams of *Chlorobium limicola*, *C. limicola f. thiosulfatophilum*, *C. vibrioforme* and *C. phaeovibrioides*. This pigment was identified as BChl 663 on basis of its absorption spectrum and estimated polarity [15]. Fig. 2 shows the absorption spectrum in acetone of purified BChl 663 from *C. limicola f. thiosulfatophilum*. Very similar spectra were obtained for BChl 663 in eluent by means of a multiwavelength detector for all five species investigated (not shown). Cochromatography of a small quantity of purified BChl 663 from *C. limicola* with the extracted pigments from *P. aestuarii* gave identical elution times for the BChls 663 from these two species. In view of the spectral identity and the nearly identical elution times of BChl 663 in the chromatograms of the remaining species, it is, therefore, assumed that this pigment is identical for all species of green sulfur bacteria studied. BChl 663, however, is clearly not identical to the primary electron acceptor of heliobacteria, 8^1-hydroxy Chl *a* [20], in view of its much lower polarity. It was checked that BChl 663 was the only pigment with an absorption band near 660–670 nm in *C. limicola* which eluted within the first 90 min.

The absorption spectra of BChl 663 and of BPhe 663 (Fig. 2) were strikingly similar to those of Chl *a* and Phe *a*, respectively (see also Table I). This indicated that the resonant parts of the porphyrin macrocycle of BChl 663 and Chl *a* are probably identical. The CD spectrum of BPhe 663 in benzene (Fig. 3) was very similar to that of Phe *a*, and clearly different from that of the 13^2-epimer [26]. The same applied to BChl 663 in relation to Chl *a* (not shown). BChl 663, thus, has the same stereochemical configuration as Chl *a* from plants. The absorption spectrum of BChl 663 differs from that obtained by Braumann et al. [15], but the spectra of the pheophytins agree well.

BChl 663 showed a strong tendency for pheophytinization. As will be discussed below, pheophytinization did not occur or only to a limited extent during analytical HPLC, but, at least in our hands, it could not be avoided during the preparative scale isolation and subsequent handling of this pigment from *P. aestuarii* and *C. limicola*. Also, solutions of BPhe 663 were found to show rapid epimerization. The equilibrium constant for epimerization, [BPhe 663]/[BPhe 663'], was determined to be 3.3 in acetone. In our chromato-

TABLE I

Spectroscopic parameters for BChl 663, BPhe 663, Chl a and Phe a

The numbers in parentheses represent the relative intensities.

Solvent		Chl *a*	BChl 663	Phe *a*	BPhe 663
Acetone	λ^{red}_{max} (nm)	661.6 (81)	662.0 (71)	665.6 (45)	665.4 (45)
	λ^{blue}_{max} (nm)	429.8 (100)	430.4 (100)	408.8 (100)	408.8 (100)
Diethyl ether	λ^{red}_{max} (nm)	660.3 (78)	660.4 (70)	666.8 (50)	666.8 (50)
	λ^{blue}_{max} (nm)	428.4 (100)	428.8 (100)	408.2 (100)	408.0 (100)
Benzene	λ^{red}_{max} (nm)	665.4 (79)	664.8 (68)	670.0 (49)	670.2 (49)
	λ^{blue}_{max} (nm)	432.5 (100)	432.4 (100)	414.0 (100)	414.2 (100)

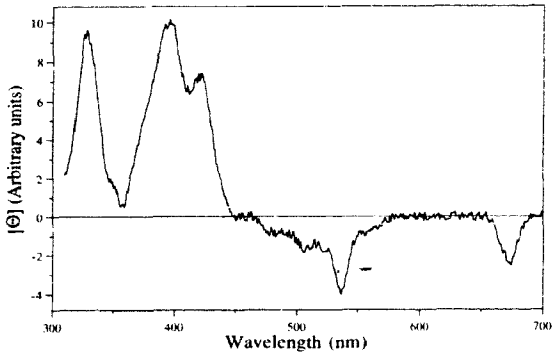

Fig. 3. Circular dichroism spectrum of PBhe 663 in benzene.

graphic system it was possible to separate the two epimers (Fig. 4), also on preparative scale, but during the subsequent handling the pigments rapidly converted into an epimer mixture. Although we were unable to determine the exact rate constants of epimerization, we estimate that they must be at least an order of magnitude higher than for Phe *a* [27]. BChl 663 showed no epimerization under the same experimental conditions. The existence of epimers of BPhe 663 shows that BChl 663 is not a derivative of BChl *c* as earlier proposed [15], since BPhe *c*, which has two protons at C-13^2, cannot form epimers.

By means of cochromatography of an epimeric mixture of purified BPhe 663 with an extract of *P. aestuarii*, we were able to identify the peaks at 13 and 10 min in the elution diagram (Fig. 1) as BPhe 663 and its 13^2-epimer, BPhe 663′, respectively. The same bands were observed in extracts from *C. limicola*. We assume that these pigments are artifacts which were either present in the cells or were produced during sonication

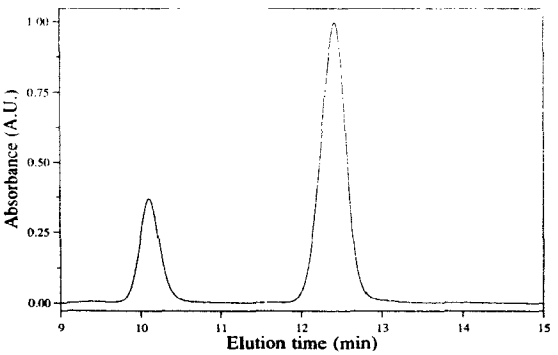

Fig. 4. Elution pattern of purified BPhe 663 from *P. aestuarii* containing peaks of the two epimeric forms, monitored at 663 nm. Chromatography of either peak resulted in the same peak ratios as displayed here, due to rapid epimerization.

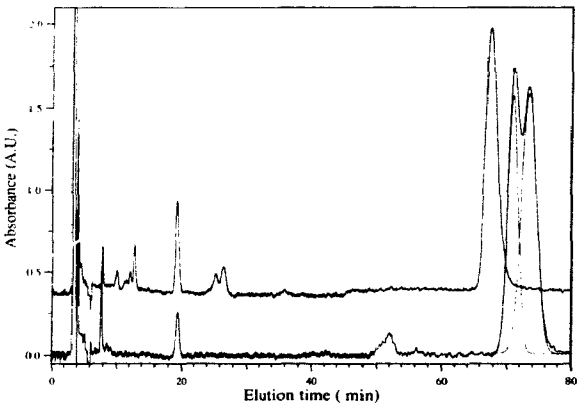

Fig. 5. Elution patterns of *C. phaeovibrioides* (lower trace) and *P. aestuarii* (upper trace) monitored at 390 nm. Peaks in the time range of 2–10 min are carotenoids. The peak near 19 min is BChl 663. The peak at 68 min in the elution profile of *P. aestuarii* is BChl *a*. The peak at 73 min in the chromatogram of *C. phaeovibriodes* is BChl *b*, presumably a contamination (see text). The BChl *a* and BChl *b* peaks were resolved into two gaussians (broken lines); the BChl *a* peaks were normalized. The nature of the minor peaks at 26 min was not further investigated. A.U., arbitrary units.

of the cells. No BPhe 663 was detected in the other species tested.

BChl 663 contents in various species

Fig. 5 shows typical elution patterns of *P. aestuarii* and *C. phaeovibrioides*, monitored at 390 nm. At this wavelength not only the peaks of BChl 663, but also those of carotenoids and of BChl *a* are clearly visible. The various homologs of the chlorosome pigments BChl *c* and *e* eluted in the time range 2–5 h (not shown). The first pigments to be eluted were the strongly apolar carotenoids (isorenieratene and β-isorenieratene in *C. phaeovibrioides* [28]; chlorobactene in *P. aestuarii* and the other species [29]). Minor amounts of more polar carotenoids eluted at somewhat longer elution times. The peak at 67 min in the elution pattern of *P. aestuarii* was identified as BChl *a* on basis of its absorption spectrum. The same peak is visible at much reduced height in the elution pattern monitored at 663 nm (Fig. 1). By cochromatography with an extract of the purple bacterium *Rhodobacter sphaeroides* G1C and by monitoring the elution pattern at 750 nm additionally, it was established that in *P. aestuarii* only BChl *a* esterified with phytol was present, as was confirmed by mass spectrometry (data not shown).

Single elution peaks at about 70 min were also obtained with extracts of *C. limicola* and *C. limicola f. thiosulfatophilum*. Although there is no doubt on the spectroscopic BChl *a* characteristics of these peaks (by monitoring at 750 nm) and the identity of phytol as the

esterifying alcohol [30], there still is a minor possibility that the BChl *a*-like pigments in these species studied are not identical. For *C. phaeovibrioides* and also for some samples of *C. vibrioforme* an extra peak at somewhat longer elution time than BChl *a* was observed (see Fig. 5), which was identified as BChl *b* by means of its absorption spectrum, indicating that these cultures suffered from a small contamination with a BChl *b*-containing purple bacterium. The band at 51 min in the chromatogram of *C. phaeovibrioides* was identified by means of its absorption spectrum as 2-desvinyl-2-acetyl Chl *a*, a well-known conversion product of BChl *a*. A similar peak was sometimes observed in the elution diagrams of *C. limicola* and *C. limicola f. thiosulphatophilum* as reported earlier [16], but not in the other species.

Table II shows the BChl 663 content for the various species, calculated from the areas of the corresponding elution peaks (monitored at 390 nm) with respect to the BChl *a* peak. When necessary, the BChl *a* contents were corrected for the presence of 2-desvinyl-2-acetyl Chl *a*. The BChl 663 contents varied between 11 and 33 BChls *a* per BChl 663. *P. aestuarii* contained about 13 BChls *a* per BChl 663, which is in good agreement with the results of Braumann et al. [15] on isolated and solubilized membranes. The variation by roughly a factor of three in the BChl 663 : BChl *a* ratio for the different preparations does not necessarily imply different pigment compositions or structures of the core complexes in the various species studied. Since BChl *a* is present in the core complex as well as in the BChl *a* protein [6,7] and in the chlorosomes [2–5], the different pigment ratios may also reflect a different ratio of the amount of core complex with respect to these antenna components. Measurements of the BChl *a* to reaction center ratio, by measuring the amount of photo-oxidizable primary electron donor, *P*-840, will be needed to resolve this point.

TABLE II

BChl 663 content of several green sulfur bacteria

For each species the molar ratio of BChl *a* is given. Unless otherwise stated, each value is an average of three measurements. The following molar extinction coefficients in eluent were used for the calculation of the molar ratios: BChl *a* = 90 $mM^{-1}cm^{-1}$ (at 773 nm) [31], BChl 663 = 85 $mM^{-1}cm^{-1}$ (at 662.5 nm, assumed to be equal to Chl *a* in eluent), 2-desvinyl-2-acetyl Chl *a* = 65.2 $mM^{-1}cm^{-1}$ (at 667 nm) [32]. The values were recalculated to the extinction coefficients at 390 nm from the corresponding absorption spectra.

Species	BChl 663 content
P. aestuarii [a]	0.08 ± 0.02
C. limicola	0.03 ± 0.005
C. vibrioforme [b]	0.09 ± 0.02
C. phaeovibrioides [b]	0.05 ± 0.01
C. limicola f. thiosulfatophilum	0.07 ± 0.005

[a] Average of 5 measurements.
[b] Peak areas determined by gaussian deconvolution.

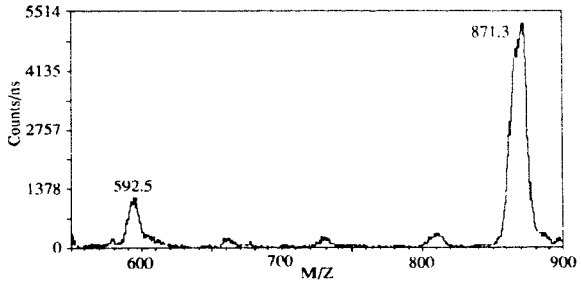

Fig. 6. The high-mass part of a ^{252}Cf-PDMS spectrum of BPhe 663.

Spectroscopic identification

As mentioned earlier, optical spectroscopy of BChl 663 and its pheophytin indicated a strong similarity between BChl 663 and Chl *a*. ^{252}Cf-plasma desorption mass spectrometry revealed no differences. The mass spectrum (Fig. 6) showed that BPhe 663 has the same molecular mass as Phe *a*, while the mass difference between the molecular ion and the first large fragment at 592.5 mass units indicates that the esterifying alcohol is phytol, as with plant Phe *a* and Chl *a*, in agreement with results reported earlier [15]. To establish possible differences between BChl 663 and Chl *a*, cochromatography of purified Chl *a* with an extract of *P. aestuarii* was performed, as well as of purified BPhe 663 with Phe *a*. Both experiments showed slight differences in elution time between the corresponding pigments. Therefore, ^1H-NMR was performed on both BPhe 663 and Phe *a*, in an attempt to find the isomeric difference between the two pigments.

^1H-NMR was, however, complicated by two factors: The amount of BPhe 663 available was low to NMR standards and, as mentioned earlier, solutions of BPhe 663 were found to show rapid epimerization. As reported before [25], the ^1H-NMR spectra of epimers are rather different, especially with respect to the protons on the esterifying alcohol and the protons attached to rings IV and V (Fig. 7). Although this effect may be present to a lesser extent in (bacterio)pheophytins when compared to (bacterio)chlorophylls [33], it may still obscure small differences due to other substitutions on the rings. Therefore, the ^1H-NMR spectrum of BPhe 663 was compared to that of an epimeric mixture of Phe *a* (Figs. 8 and 9). The Phe *a*/*a'* and BChl 663/663' ^1H-NMR lines were assigned according to Ref. 33 and are not listed here for that reason.

The ^1H-NMR, as well as the COSY spectrum, showed a strong resemblance between Phe *a* and BPhe 663, establishing the presence of vinyl- and ethyl-groups on the BPhe 663 macrocycle. In both spectra the lines

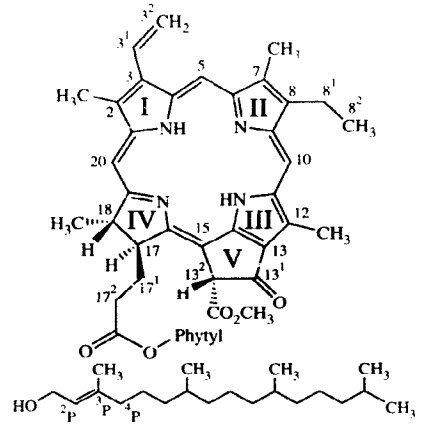

Fig. 7. Molecular structure of Phe *a*. Exchange of the 13^2-proton and CO_2CH_3-group produces the epimer. Phytol (bottom) is the esterifying alcohol.

of the C-5, C-10 and C-20 protons were present, demonstrating the absence of substituents on these carbon atoms. Since the total molecular mass and that of the esterifying alcohol are identical for both BPhe 663 and Phe *a* and there is no difference in the resonant part of the macrocycle, the anticipated difference must be in the substituents on the macrocycle. Also, the isomeric difference obviously causes the epimerization rates to differ to a considerable extent and therefore most likely involves substitutions on rings IV and/or V. In Figs. 8 and 9, the most notable difference in the ^{1}H-NMR spectra is marked by an arrow. The COSY spectrum of the Phe *a*/*a'* epimeric mixture (not shown) clearly indicated a coupling of a peak at 2.8 ppm with a peak at 5.3 ppm. In the BChl

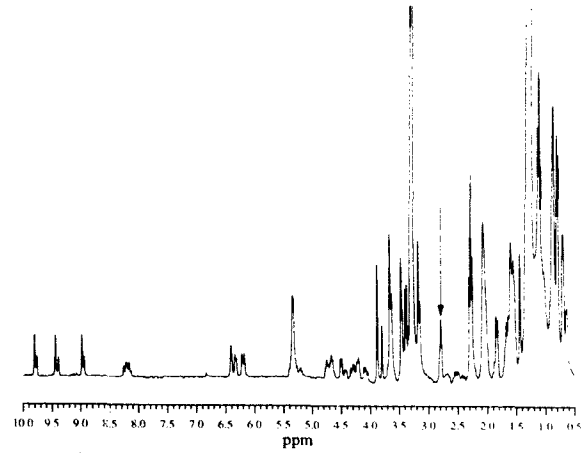

Fig. 8. ^{1}H-NMR spectrum of a Phe *a*/*a'* epimeric mixture in [$^2H_6$]acetone at 240 K. The arrow marks the main difference between this ^{1}H-NMR spectrum and that of BPhe 663.

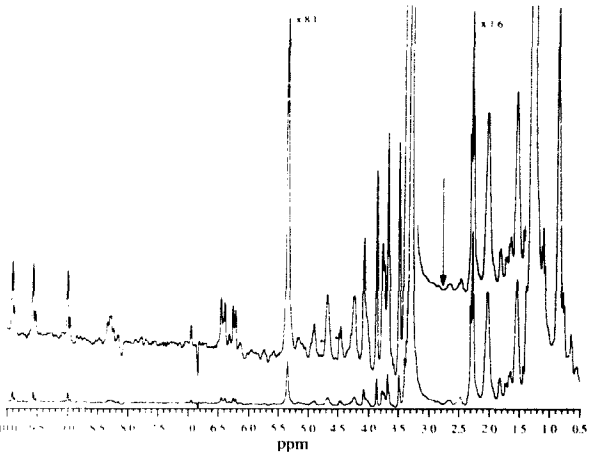

Fig. 9 ^{1}H-NMR spectrum of a BPhe 663/663' epimer mixture in [$^2H_6$]acetone, 240 K.

663/663' spectrum, however, the first line was absent, and the coupling of the latter one was replaced by one at 2.0 ppm. From a straigh*forward interpretation of the Phe *a*/*a'* ^{1}H-NMR data, the peak at 2.8 ppm most likely involves the 17^1-CH_2 group (Fig. 7) [33]. The absence of this peak in the BPhe 663-spectrum could indicate the lack of one CH_2-group in the propionic-acid chain on ring IV, which would very likely give rise to a changed epimerization behavior with respect to Phe *a*. Another suggestion would involve an isomerization in the phytyl chain, shifting the ^{2}P-^{3}P double bond to the ^{3}P-^{4}P position (Fig. 7). For the assignment of these kinds of differences, however, long-range couplings have to be studied, which implies the need of a larger amount of sample. Also, as far as we know, the chlorophyll biosynthetic pathways do not support either kind of substitution [34]. Further analysis will be needed for a final structural assignment.

Conclusions

The object of this study was to identify the 670 nm pigment present in cells, membranes and solubilized fractions of membranes of green sulfur bacteria, which is believed to function as the primary electron acceptor [17–19]. The previously developed successful methods of extraction and separation [20,25] were used to investigate the pigment composition of five species of green sulfur bacteria, again with the exclusion of artifact formation during extraction and subsequent chromatographic analysis. The only likely candidate for the primary electron acceptor is BChl 663, a pigment eluting in the polarity range between the apolar carotenoids and BChl *a* [15,16]. In this communication, we show that BChl 663 is an isomer of Chl *a*, the major pigment

of oxygenic photosynthesis. The primary electron acceptors of PS I, heliobacteria and green sulfur bacteria are, thus, found to be closely related.

Pigment analysis studies on green sulfur bacteria usually suffer from a high rate of pheophytinization, either during the preparation of membranes or solubilized fractions and/or during the extraction and subsequent chromatographic analysis [15]. Although our fast analytical scale extraction and analysis methods do not appear to suffer from these drawbacks, the collection and purification of BChl 663 on preparative scale, at least in our hands, inevitably produced the corresponding pheophytin. Nevertheless, sufficient amounts of BChl 663 could be obtained to demonstrate the spectroscopic similarity between BChl 663 and Chl *a*, and to present evidence by means of CD-spectroscopy, that BChl 663 is the 'normal' $13^2(R)$-epimer species. Although the molecular masses of BChl 663 and Chl *a* were found to be the same, the elution times and NMR spectra showed that these pigments are not identical. Mainly due to the rapid pheophytinization of BChl 663 and the rapid epimerization of its pheophytin, a definite assignment of the structure of BChl 663 is not possible at this time. All membrane-associated (bacterio)chlorophylls of green sulfur bacteria appear to be exclusively esterified with phytol, in contrast to the chlorosomal BChls *c*, *d* and *e*. This indicates a partially different biosynthetic pathway.

Even more so than with heliobacteria, the size of the photosynthetic unit of green sulfur bacteria is uncertain. The BChl *a*-content of chlorosomes of *C. limicola* has been found to be around 1% [2], yielding 15–20 BChls *a*/RC, whereas the membrane and the associated BChl *a* proteins together probably contain about 80 BChls *a*/RC [12]. This results in a total of about 100 BChls *a*/RC. This number is uncertain, because the differential extinction coefficient of *P*-840/*P*-840$^+$ is not known and, moreover, it could well be variable, depending on growth conditions and species studied. In this study, the BChl 663 content was shown to be between 33 and 11 BChls *a*/BChl 663, indicating the presence of between 3 and 9 BChls 663/RC. Since not all of these pigments can be functioning as the primary electron acceptor, the role of the remaining BChls 663 in the core-complex remains unknown. Experiments are under way to determine the amount of BChl 663 in an isolated core complex. The results presented here and elsewhere [15] show, however, that it is highly unlikely that another 670 nm species functions as the primary electron acceptor in green sulfur bacteria.

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References

1 Amesz, J. (1991) in Variations in Autotrophic Life (Shively, J.M. and Barton, L.L. eds.) pp. 99–119, Academic Press, London.
2 Gerola, P.D. and Olson, J.M. (1986) Biochim. Biophys. Acta 848, 69–76.
3 Van Dorssen, R.J., Gerola, P.D., Olson, J.M. and Amesz, J. (1986) Biochim. Biophys. Acta 848, 77–82.
4 Stolz, J.F., Fuller, R.C. and Redlinger, T.E. (1990) Arch. Microbiol. 154, 422–427.
5 Otte, S.C.M., Van der Heiden, J.C., Pfennig, N. and Amesz, J. (1991) Photosynth. Res. 28, 77–87.
6 Olson, J.M. (1981) Biochim. Biophys. Acta 549, 33–51.
7 Matthews, B.E., Fenna, R.E., Bolognesi, M.C., Schmid, M.F. and Olson, J.M. (1979) J. Mol. Biol. 131, 259–285.
8 Fowler, C.F., Nugent, N.A. and Fuller, R.C. (1971) Proc. Natl. Acad. Sci. USA 68, 2278–2282.
9 Whitten, W.B., Pearlstein, R.M. and Olson, J.M. (1979) Photochem. Photobiol. 29, 823–828.
10 Staehelin, L.A., Golecki, J.R. and Drews, G. (1980) Biochim. Biophys. Acta 589, 30–45.
11 Nitschke, W. and Rutherford, A.W. (1991) Trends Biochem. Sci. 16, 241–245.
12 Swarthoff, T. and Amesz, J. (1979) Biochim. Biophys. Acta 548, 427–432.
13 Vasmel, H., Swarthoff, T., Kramer, H.J.M. and Amesz, J. (1983) Biochim. Biophys. Acta 725, 361–367
14 Hurt, E.C. and Hauska, G. (1984) FEBS Lett. 168, 149–154.
15 Braumann, T., Vasmel, H., Grimme, L.H. and Amesz, J. (1986) Biochim. Biophys. Acta 848, 83–91.
16 Watanabe, T. and Kobayashi, M. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.) Vol. II, pp. 109–112, Kluwer, Dordrecht.
17 Nuijs, A.M., Vasmel, H., Joppe, H.L.P., Duysens, L.N.M. and Amesz, J. (1985) Biochim. Biophys. Acta 807, 24–34.
18 Shuvalov, V.A., Amesz, J. and Duysens, L.N.M. (1986) Biochim. Biophys. Acta 851, 1–5.
19 Van Kan, P.J.M. (1991) Ph.D. Thesis, University of Leiden.
20 Van de Meent, E.J., Kobayashi, M., Erkelens, C., Van Veelen, P.A., Amesz, J. and Watanabe, T. (1991) Biochim. Biophys. Acta 1058, 356–362.
21 Olson, J.M. (1978) Int. J. Syst. Bacteriol. 28, 128–129.
22 Holt, S.C., Conti, S.F. and Fuller, R.C. (1966) J. Bacteriol. 91, 311–323.
23 Gloe, A., Pfennig, N., Brockmann, H., Jr. and Trowitzsch, W. (1975) Arch. Microbiol. 102, 103–109.
24 Larsen, H. (1953) On the Microbiology and Biochemistry of the Photosynthetic Green Sulfur Bacteria, Kgl. Norske Videnskab. Selskabs Skrifter No. 1, Trondheim.
25 Kobayashi, M., Van de Meent, E.J., Erkelens, C., Amesz, J., Ikegami, I. and Watanabe, T. (1991) Biochim. Biophys. Acta. 1057, 89–96.
26 Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M. and Saitoh, S. (1984) Anal. Chem. 56, 251–256.
27 Watanabe, T., Nakazato, M. and Honda, K. (1986) Chem. Lett. 253–256.
28 Liaaen-Jensen, S. (1965) Acta Chem. Scand. 19, 1025–1030.
29 Schmidt, K. and Schibarr... (1970) Arch. Mikrobiol. 74, 350–355.

30 Gloe, A. and Pfennig, N. (1974) Arch. Mikrobiol. 96, 93–101.
31 Hoff, A.J. and Amesz, J. (1991) in Chlorophylls (Scheer, H., ed.) pp. 723–738, CRC Press, Boca Raton.
32 Lindsay Smith, J.R. and Calvin, M. (1966) J. Am. Chem. Soc. 88, 4500–4506.
33 Smith, K.M., Goff, D.A. and Abraham, R.J. (1984) Org. Magn. Res. 22, 779–783.
34 Leeper, F.J. (1991) in Chlorophylls (Scheer, H., ed.) pp. 407–431, CRC Press, Boca Raton.