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Red Shift of Absorption Maxima in Chlorobiineae through Enzymic Methylation of Their Antenna Bacteriochlorophylls†

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ABSTRACT: The bacteriochlorophyll *d* producing photosynthetic green sulfur bacteria *Chlorobium vibrioforme* forma *thiosulfatophilum* strain NCIB 8327 and *C. vibrioforme* strain Bl-20 respond to reduced light conditions in culture by performing methylations at the 4- and 5-substituents, for example, converting the 4-Et into 4-*n*-Pr, 4-*i*-Bu, and even 4-neoPn. During this process, the absorption maximum in living cells of *C. vibrioforme* strain Bl-20 red shifts from 714 to about 728 nm. Eventually, the *C. vibrioforme* forma *thiosulfatophilum* strain NCIB 8327 culture carries out a δ-methylation to produce the bacteriochlorophylls *c* (λ_{max} ca. 750 nm); the new UC Davis bacteriochlorophyll *c* culture is named *C. vibrioforme* forma *thiosulfatophilum* strain D. It is possible that the homologation process increases hydrophobic interactions between individual BChl molecules, giving rise to larger aggregates in the antenna system. Alternatively, the additional methyl units attached to the 4-position shift the absolute configuration of the 2-(1-hydroxyethyl) group from pure *R* in the case of 4-Et to pure *S* in the case of 4-neoPn, which in turn might determine the size of the in vivo aggregates due to the intrinsic nature of the pigment protein system. It is suggested that the bacteriochlorophylls *c* from *Chloroflexus aurantiacus* strain J-10-fl and the bacteriochlorophylls *e* from *Chlorobium phaeovibrioides* might have undergone similar meso methylation as a response to external environmental pressure such as low light intensity.

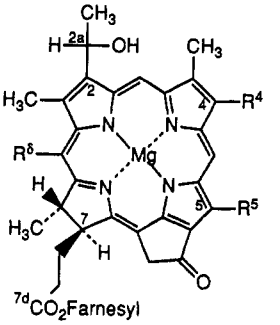
The structural assignments of the bacteriochlorophylls *d* (BChl-d)[1] were made by Holt in the 1960s after classical oxidative degradation (Holt & Hughes, 1961; Hughes & Holt, 1962; Holt & Purdie, 1965). Over the years some alterations to Holt's assignments have been made, mostly with regard to stereochemistry; the currently accepted structures (Smith & Goff, 1985) for the BChl-d homologous mixture are shown in **1** (Table I). The structures of the BChl-c were the subject of considerably more discussion, but their finalized structures (Smith et al., 1983a) are depicted in **2** (Table I).

The most novel features in the structures of the BChl-c and BChl-d are the extra methylation attached to the 4 and 5 side chains and the δ-methyl substituent (BChl-c only). Soon after the gross structural assignments were made (Holt & Purdie, 1965; Kenner et al., 1976), efforts were concentrated on establishing the origin of the extra methyl units. Carbon-13 labeling experiments showed that the extra methyl groups in the BChl-c from *Prosthecochloris aestuarii* (formerly *Chloropseudomonas ethylica*) [i.e., the δ-methyl, the terminal carbons of the isobutyl (*i*-Bu) and *n*-propyl (*n*-Pr) at position 4 and of the ethyl at position 5] were all derived from L-methionine (Kenner et al., 1976, 1978).

Experimental Procedures

General. Proton NMR spectra were obtained in $CDCl_3$ at 360 MHz (Nicolet NT360) with chemical shifts reported in parts per million relative to internal chloroform (7.258 ppm, 300 MHz). *Chlb* strain D was cultured and the Bmphs were

Table I: Structural Assignments for the BChl-d (**1**) and BChl-c (**2**)



	cmpd	Holt's band no.	R^4	R^5	R^8	configuration at C-2a
BChl-d	**1a**	6	Et	Me	H	*R*
	1b	4	Et	Et	H	*R*
	1c	5	*n*-Pr	Me	H	*R*
	1d	2	*n*-Pr	Et	H	*R*
	1e	3	*i*-Bu	Me	H	*S*
	1f	1	*i*-Bu	Et	H	*S*
	1g		neoPn	Me	H	*S*
	1h		neoPn	Et	H	*S*
BChl-c	**2a**	6	Et	Me	Me	*R*
	2b	5	Et	Et	Me	*R*
	2c	4	*n*-Pr	Et	Me	*R*
	2d	3	*n*-Pr	Et	Me	*S*
	2e	2	*i*-Bu	Et	Me	*R*
	2f	1	*i*-Bu	Et	Me	*S*

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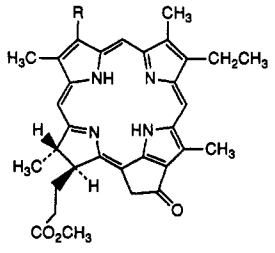
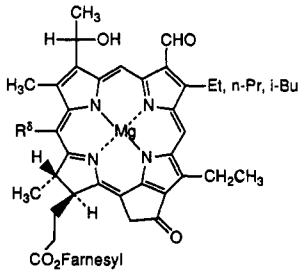
isolated and purified as described previously (Smith & Goff, 1985; Huster & Smith, 1989) for *Chlb* 8327. Cells were grown under anaerobic conditions (CO_2 gas) in 20-L carboys (11-in. diameter × 10 in. high, excluding neck) at 30 (±2) °C in front of a bank of six 48-in. soft white 40-W fluorescent tubes; the center of the carboy was 14–18 in. from the closest tube, and the medium was stirred gently with a 1-in. magnetic stirrer bar. The pigments were archived/stored at −40 °C in the form of solid foams of the corresponding Bmph homologous mixtures. *Chlfl* J-10-fl culture and frozen cells were obtained from Professor R. E. Blankenship (Arizona State University). The bacteria were cultivated at 55 °C in the medium of Pierson and Castenholz (1974); the *Chloroflexus* BChl-c were isolated, demetalated, and transesterified to form the Bmph-c and then purified and examined by HPLC as described previously (Kenner et al., 1978; Smith et al., 1980a,b, 1983a). *P. aestuarii* was grown as described elsewhere (Kenner et al., 1978), and the BChl-c **2** were isolated, demetalated, and transesterified to form the Bmph-c and then purified and examined by HPLC as described previously (Kenner et al., 1978; Smith et al., 1980a,b, 1983a). Precise HPLC conditions are noted in the figure captions. [4-Et,5-Me]-Bmph-d **3** was



3 R = CH(OH)CH$_3$
4 R = CH=CH$_2$



5 R = CH$_3$
6 R = H

synthesized as previously described (Smith et al., 1980a,b) by HBr/acetic acid treatment of methyl pyropheophorbide *a* (**4**), which was in turn obtained from *Spirulina maxima* alga (Smith et al., 1985) via methyl pheophorbide *a*. The product was identical in all respects with an authentic sample (Smith et al., 1980a).

Results

Incubation of Chlb 8327 with L-[^{13}C-CH_3]*Methionine.* During the course of biosynthetic work on the BChl-d (**1**) from *Chlb* 8327, it was decided to incubate the growth medium with L-[^{13}C-CH$_3$]methionine and then to study the resulting methyl bacteriopheophorbides *d* (**3**, Bmph-d) by carbon-13 NMR spectroscopy. The labeled methionine was prepared in 79% yield by reductive methylation of L-homocystine in presence of [^{13}C]methyl iodide (Kenner et al., 1978). In 1984, a 1-day-old 20-L batch of the *Chlorobium* culture, grown continuously in Davis for 4 years (subsequent to its use for structural identification of its Bmph in 1980), was incubated with 200 mg of L-[^{13}C-CH$_3$]methionine, and after 2 weeks under fluorescent lights with minor stirring (magnetic stirrer bar) at 34 °C, the cells were harvested by centrifugation.

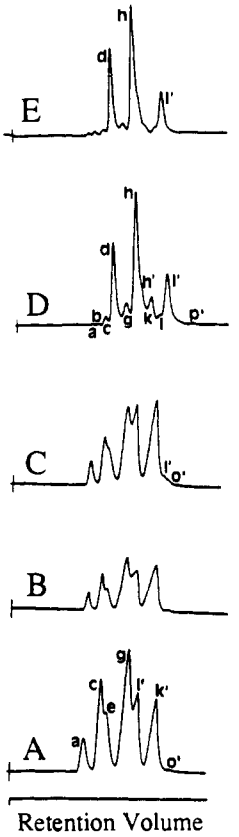


FIGURE 1: HPLC chromatograms of the natural mixture (i.e., 5-Me and 5-Et combined) of Bmphs isolated from *Chlb* in (A) 1980 (*Chlb* 8327), (B) 1981, (C) 1982, (D) 1983, and (E) 1984 (*Chlb* strain D). Peak assignments: (a) [4-Et,5-Me,δ-H]; (b) [4-Et,5-Me,δ-Me]; (c) [4-Et,5-Et,δ-H]; (d) [4-Et,5-Et,δ-Me]; (e) [4-*n*-Pr,5-Me,δ-H]; (f) [4-*n*-Pr,5-Me,δ-Me]; (g) [4-*n*-Pr,5-Et,δ-H]; (h) [4-*n*-Pr,5-Et,δ-Me]; (i) [4-*i*-Bu,5-Me,δ-H]; (j) [4-*i*-Bu,5-Me,δ-Me]; (k) [4-*i*-Bu,5-Et,δ-H]; (l) [4-*i*-Bu,5-Et,δ-Me]; (m) [4-neoPn,5-Me,δ-H]; (n) [4-neoPn,5-Me,δ-Me]; (o) [4-neoPn,5-Et,δ-H]; (p) [4-neoPn,5-Et,δ-Me]. Pigments all have *R* absolute stereochemistry at 2a unless primed (e.g., h′) to indicate *S* absolute stereochemistry at 2a. HPLC conditions: Waters Associates μBondapak reversed-phase cartridge, Z-module, 2.6 mL/min of 85:15 methanol/water; Perkin-Elmer LC55B variable-wavelength detector set at 650 nm. The complete horizontal scale in (A) represents a total elution volume of approximately 125 mL.

Pigments were extracted with methanol, and after treatment with methanol/sulfuric acid and chromatography, the appropriate Bmph were isolated.

Carbon-13 NMR spectra for each of the individual HPLC-separated homologues (supposed Bmph-d) possessed, in every case, one heavily enriched carbon-13 more than anticipated on the basis of the known structures **3** for the Bmph-d. In every case, an enriched carbon resonance which could readily be assigned to a δ-methyl (BChl-c!) substituent was apparent at about 20.4 ppm (Smith & Bobe, 1987). Optical spectroscopy on living cells (not shown) confirmed that the bacteria were indeed producing the BChl-c (**2**, λ_{max} ca. 750 nm) rather than the BChl-d (**1**, λ_{max} ca. 730 nm) (see Table I). Since the 1984 culture derived from *Chlb* 8327 was clearly different from 8327, it was therefore designated *Chlb* strain D, to indicate its origin in UC Davis.

Earlier studies had already revealed that the pigment composition of the Davis *Chlb* 8327 culture was changing. An initial roughly 3:1 ratio of the 5-Et series vs 5-Me series (Smith & Goff, 1985) appeared to have changed over a period of 4 years to a ratio of about 16:1 (spectrophotometry) in favor of the 5-Et homologues, indicating increased methylation activity in the bacterium. Fortunately, sufficient material had been stored which had been isolated from this strain between

[1] Abbreviations: BChl, bacteriochlorophyll; Chl, chlorophyll; *Chlb* 8327, *Chlorobium vibrioforme* forma *thiosulfatophilum* strain NCIB 8327; *Chlb* strain D, the *C. vibrioforme* forma *thiosulfatophilum* strain, derived from *Chlb* 8327, which now produces substantially BChl-c instead of BChl-d; *Chlfl* J-10-fl, *Chloroflexus aurantiacus* strain J-10-fl; SAM, *S*-adenosylmethionine; *n*-Pr, *n*-propyl; *i*-Bu, isobutyl; neoPn, neopentyl; ALA, δ-aminolevulinic acid; Bmph, methyl bacteriopheophorbide; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.

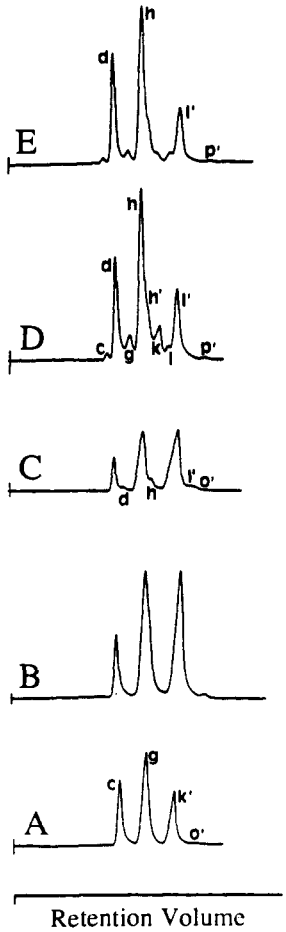


FIGURE 2: HPLC chromatograms of the [5-Et]-Bmphs separated from *Chlb* in (A) 1980 (*Chlb* 8327), (B) 1981, (C) 1982, (D) 1983, and (E) 1984 (*Chlb* strain D). Peak assignments: (c) [4-Et,5-Et,δ-H]; (d) [4-Et,5-Et,δ-Me]; (g) [4-*n*-Pr,5-Et,δ-H]; (h) [4-*n*-Pr,5-Et,δ-Me]; (j) [4-*i*-Bu,5-Me,δ-Me]; (k) [4-*i*-Bu,5-Et,δ-H]; (l) [4-*i*-Bu,5-Et,δ-Me]; (o) [4-neoPn,5-Et,δ-H]; (p) [4-neoPn,5-Et,δ-Me]. Pigments all have *R* absolute stereochemistry at 2a unless primed (e.g., h′) to indicate *S* absolute stereochemistry at 2a. HPLC conditions as in Figure 1.

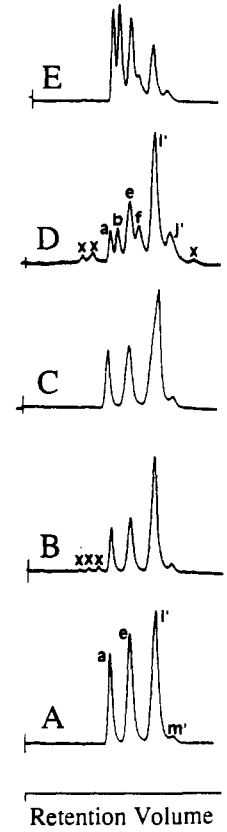


FIGURE 3: HPLC chromatograms of the [5-Me]-Bmphs separated from *Chlb* in (A) 1980 (*Chlb* 8327), (B) 1981, (C) 1982, (D) 1983, and (E) 1984 (*Chlb* strain D). Peak assignments: (a) [4-Et,5-Me,δ-H]; (b) [4-Et,5-Me,δ-Me]; (e) [4-*n*-Pr,5-Me,δ-H]; (f) [4-*n*-Pr,5-Me,δ-Me]; (i) [4-*i*-Bu,5-Me,δ-H]; (j) [4-*i*-Bu,5-Me,δ-Me]; (m) [4-neoPn,5-Me,δ-H]. Pigments all have *R* absolute stereochemistry at 2a unless primed (e.g., h′) to indicate *S* absolute stereochemistry at 2a. x indicates an impurity. HPLC conditions as in Figure 1.

1980 and 1984 that a measure of these time-dependent changes was available by way of reversed-phase HPLC analysis. Figures 1–3 show the appropriate HPLC traces; peak identities (legend, Figure 1) were established by extensive HPLC co-injection analysis (not shown).

The *Chlb* 8327 culture was originally obtained in 1980 and was grown continuously in either 4.6- or 20-L batches; it was subcultured after periods varying between 10 and 200 days, with particularly long subculture periods in 1983 and 1984. Our structural studies (Smith & Goff, 1985) were carried out entirely on stockpiled 1980 (*Chlb* 8327) material. As early as 1981 a change in pigment composition occurred (Figures 1B, 2B, 3B) in which more of the 4-*i*-Bu homologues were produced relative to the other bands (4-Et, 4-*n*-Pr; Figures 1A, 2A, 3A). Thus, a trend toward increased methylation was even then apparent. Two years after initial growth of the strain (Figures 1C, 2C, 3C), additional peaks appeared in the HPLC tracings of the 5-Et-Bmph (Figure 2C), but *not* in the case of the 5-Me-Bmph (Figure 3C). A dramatic change occurred in 1983 (possibly as a result of a 200-day subculture period) for both series, although more pronounced in the 5-Et series (Figure 2D); the latter had experienced an almost complete switch to Bmph-c with increased retention time due to the presence of one additional methyl group. Furthermore, additional shoulders (Figure 4B) indicated the presence of 2a-(*S*)-[5-Pr;4-Et]- and 2a-(*R*)-[5-*i*-Bu;4-Et]-Bmph-c in a similar fashion as for the Bmph-c from *P. aestuarii* strain C.

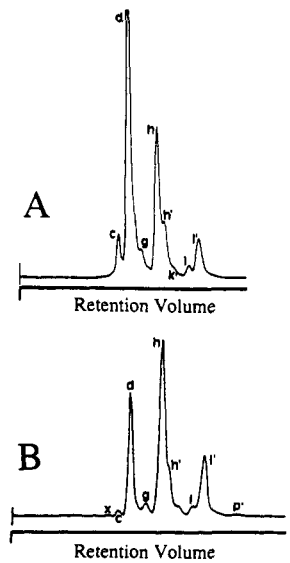


FIGURE 4: HPLC chromatograms of the natural mixture of Bmphs isolated from (A) *P. aestuarii* strain C. e., subculture 3, and (B) *Chlb* strain D in 1984 (cf. Figure 1E). Peak assignments: (c) [4-Et,5-Et,δ-H]; (d) [4-Et,5-Et,δ-Me]; (g) [4-*n*-Pr,5-Et,δ-H]; (h) [4-*n*-Pr,5-Et,δ-Me]; (k) [4-*i*-Bu,5-Et,δ-H]; (l) [4-*i*-Bu,5-Et,δ-Me]; (o) [4-neoPn,5-Et,δ-H]; (p) [4-neoPn, 5-Et,δ-Me]. Pigments all have *R* absolute stereochemistry at 2a unless primed (e.g., h′) to indicate *S* absolute stereochemistry at 2a. HPLC conditions as in Figure 1.

e., subculture 3, previously reported (Smith et al., 1983a) and reproduced in Figure 4A.

From a sample of *Chlb* strain D culture (1984) were isolated five subcultures from single cells, and these were grown in

liquid medium. The absorption spectra of subcultures 1 and 3 possessed in vivo absorption maxima at about 750 nm, whereas subcultures 2, 4, and 5 had absorption maxima at 730–732 nm (not shown). In addition to the five subcultures, a contaminant was detected which was identified as *Rhodopseudomonas palustris*, a motile BChl-a containing purple non-sulfur bacterium.

Concerned that the origin of the BChl-c in *Chlb* strain D might be due to contamination with *P. aestuarii* strain C. e. or strain SK 413 (the only other BChl-c producing bacteria ever grown in our Davis laboratories), the pigments from subcultures 3 and 5 were isolated and their HPLC profiles obtained. Subculture 5, with an in vivo absorption maximum at 730 nm, produced mainly BChl-d with small amounts of BChl-c, and its tracing (not shown) was very similar to that obtained from the *Chlb* 8327 pigments in 1980, except for the presence of the minor BChl-c contamination. Subculture 3 instead absorbed at 750 nm in vivo and gave rise to an HPLC profile containing the usual Bmph-c pigments as observed from *P. aestuarii* but with proportions of homologues resembling those found in the 1980 *Chlb* 8327 strain. Moreover, the 1980 *Chlb* 8327 and the 1984 *Chlb* strain D subculture 3 (the latter absorbing at 750 nm) were shown to be morphologically identical with each other and quite different from *P. aestuarii*. The 5-Me homologue pigments from *Chlb* strain D subculture 3 (1984) showed a completely different composition, still with Bmph-d as the major component. Interestingly, three Bmph-c were present, two of which had not previously been reported ([4-*n*-Pr,5-Me] and [4-*i*-Bu,5-Me]).

Our attention next focused on proton NMR studies of the natural mixture of 5-Et- and 5-Me-Bmph. In the material isolated in 1980 (Smith & Goff, 1985) in the meso region, as expected, the α-meso proton is farthest downfield, whereas the δ-meso resonance is farthest upfield. The following trends hold for the chemical shifts of the individual homologues (in order of decreasing chemical shift, in parts per million):

(1) α-meso resonances: 4-neoPn(S) > 4-*i*-Bu(S) > 4-*n*-Pr(S) > 4-*i*-Bu(R) = 4-Et(S) > 4-*n*-Pr(R) > 4-Et(R).

(2) β-meso resonances: 4-Et(R,S) > 4-*n*-Pr(R,S) > 4-*i*-Bu(R,S).

(3) δ-meso resonances: 4-Et(R) = 4-*n*-Pr(R) > 4-*i*-Bu(S).

Similarly, in 360-MHz proton NMR studies of a natural mixture (Smith et al., 1983a) of Bmph-c from *P. aestuarii*, the following relative chemical shifts were observed (in order of decreasing chemical shift, in parts per million):

(1) α-meso resonances: 4-*i*-Bu,5-Et(S) > 4-*n*-Pr,5-Et(S) > 4-*n*-Pr,5-Et(R) > 4-Et,5-Et(R).

(2) β-meso resonances: 4-Et,5-Et(R) > 4-*n*-Pr,5-Et(R) > 4-*n*-Pr,5-Et(S) > 4-*i*-Bu,5-Et(S).

A very small resonance (not shown) is observable at 8.45 ppm due to a δ-meso proton from Bmph-d contamination (also produced in minor amounts by this strain in the form of the corresponding BChl-d prior to demetalation and transesterification). On the basis of these findings it seems to be general that α-meso resonances experience a downfield shift with increasing number of methyl units attached to the 4-position, whereas the β-meso resonance shows the reverse effect (i.e., upfield shift with increasing methylation).

The proton NMR spectra of the meso regions of the Bmph from subculture 3 and subculture 5 are shown in parts A and B, respectively, of Figure 5. The presence of δ-methylation is readily apparent from the substantial absence of the δ-meso proton in Figure 5A. The corresponding meso-region proton NMR spectra of the separated 5-Et and 5-Me series, along with meso-proton assignments, are given for subculture 3 in Figure 6 and for subculture 5 in Figure 7. The large difference in the relative intensities of the δ-meso protons in parts A (5-Me series) and B (5-Et) of Figure 6 clearly confirms the observation from HPLC that the 5-Me series of Bmph from the "adapted" subculture 3 still consists substantially of Bmph-d, quite unlike the corresponding 5-Et subculture 3 pigments. For subculture 5, consisting mainly of Bmph-d, α-meso resonances from minor amounts of Bmph-c are clearly visible at about 9.9 ppm (Figures 5B, 7) and thus confirm the

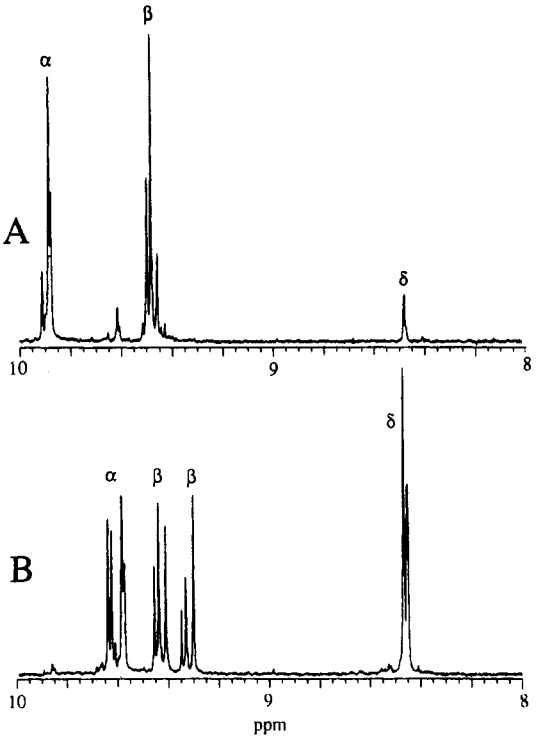


FIGURE 5: 360-MHz (Nicolet NT360) proton NMR spectra (meso region only) in $CDCl_3$ of natural mixtures of Bmph from *Chlb* strain D: (A) subculture 3; (B) subculture 5.

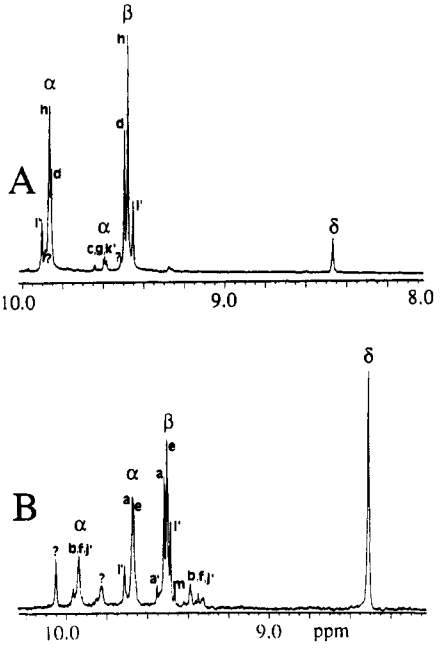


FIGURE 6: 360-MHz (Nicolet NT360) proton NMR spectra (meso region only) in $CDCl_3$ of separated Bmph from *Chlb* strain D, subculture 3: (A) [5-Et]-Bmph; (B) [5-Me]-Bmph.

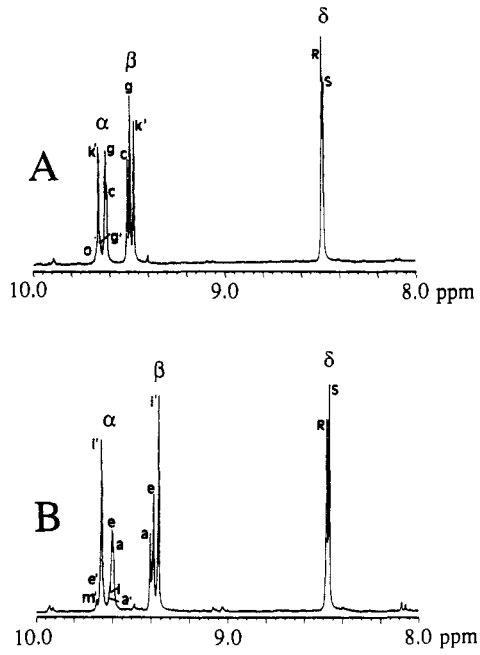


FIGURE 7: 360-MHz (Nicolet NT360) proton NMR spectra (meso region only) in $CDCl_3$ of separated Bmph from *Chlb* strain D, subculture 5: (A) [5-Et]-Bmph; (B) [5-Me]-Bmph.

interpretations from HPLC tracings.

BChl-c from C. aurantiacus Strain J-10-fl. The BChl-c composition of Chloroflexaceae consists of only the 4-Et,5-Me homologue which is esterified predominantly with stearyl alcohol at the 7d ester (Gloe & Risch, 1978; Risch et al., 1979). Isolation and HPLC analysis of the [4-Et,5-Me]-Bmph-c from *Chlfl* J-10-fl also yielded an additional peak in the HPLC tracing (Figure 8A). Isolation of this minor product via preparative HPLC provided enough product to identify the pigment as [4-Et,5-Me]-Bmph-d (**3**). The proton NMR spectrum of the product revealed *three* meso-proton resonances at 8.55, 9.54, and 9.72 ppm. This was compared with the NMR spectrum of the synthetic analogue of [4-Et,5-Me]-Bmph-*d* [prepared by treating methyl pyropheophorbide *a* (**4**) with 40% HBr/HOAc (Smith et al., 1980a,b)] and shown to be identical with **3**. In addition, simultaneous HPLC co-injections showed that the synthetic and natural products (Figure 8B) were identical. The visible spectrum of the minor product was found to be identical with that of Bmph-d which was isolated from *Chlb* 8327. Our results on the identification of the Bmph-d pigment support results of others (Brune et al., 1987) who isolated and spectrophotometrically identified small amounts of BChl-d from *Chlfl* strain J-10-fl.

The Bmph-c pigment from *Chlfl* J-10-fl has been shown (Brune et al., 1988) to consist of an approximately 50:50 mixture of the 2a-*R* and 2a-*S* diastereomers. We have confirmed this observation (Figure 8A) both from material grown in Davis and with *Chlfl* cells kindly provided by Drs. Brune and Blankenship. However, growth of *Chlfl* J-10-fl over a 12-month period in Davis has revealed that the *R* to *S* ratio can vary to as much as 70:30 (Figure 8C). This variation might possibly be attributed to the different growth conditions and reduction of light intensities.

Discussion

BChls are known to play important roles in both key processes of bacterial photosynthesis: light harvesting (by the antenna array) and light conversion (by the reaction center). The structure of the reaction center in the Chlorobiaceae

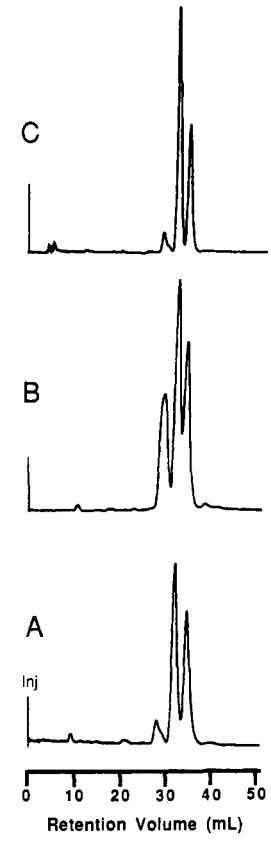


FIGURE 8: HPLC chromatograms of Bmphs isolated from *Chlfl* J-10-fl (A) natural mixture, (B) after spiking with synthetic methyl 2-(1-hydroxyethyl)pyropheophorbide *a* (**3**), and (C) after growing the culture under reduced light conditions. HPLC conditions: Waters Associates C-18 μBondapak reversed-phase cartridge, RCM 8 × 10 module, 2.0 mL/min of 85:15 methanol/water. The detector was an LDC Milton Roy SpectroMonitor D variable-wavelength detector set at 405 nm. The broadness of the spiked peak in trace B is due to the presence of approximately equal amounts of 2a-*R* and 2a-*S* components in the synthetic material **3**.

(green sulfur bacteria) is still a subject of interest (Vasmel et al., 1987; Ovchinnikov et al., 1988). Most of the pigments in green and brown anaerobic bacteria do not participate directly in the chemical process of photosynthesis, but instead act as antenna molecules, i.e., light receptors within the chlorosomes. The antenna consists of the chlorosome containing the BChl-c or BChl-d plus some BChl-a and a membrane-bound portion containing BChl-a. Energy of the absorbed light is funneled to a reaction center from where it can be introduced into photosynthesis. The exact nature and molecular arrangement of the antenna pigments, for example, in *Chlfl*, is not known but it has been suggested that 12–14 BChl-c molecules are associated in discrete aggregate rods (Wechsler et al., 1985; Betti et al., 1982). The Chloroflexaceae are thermophilic filamentous gliding bacteria which are genetically distant from the Chlorobiaceae; however, they also contain a BChl-c antenna system. The recent progress in understanding the mechanism of primary photochemistry in *Chlfl* shows that the reaction centers in this family of green bacteria are very different from those found in Chlorobiaceae, but are very similar to those found in purple bacteria (Vasmel et al., 1987). It has been established that there is an approximately 30:1 ratio of BChl-c:BChl-a, which are located in the chlorosome and in the membrane (BChl-a).

It is apparent from the information presented above that every strain of anaerobic green bacteria grown in this laboratory has experienced, in the past, a pigment change toward increased alkylation at the 4-, 5-, and δ-meso positions, with

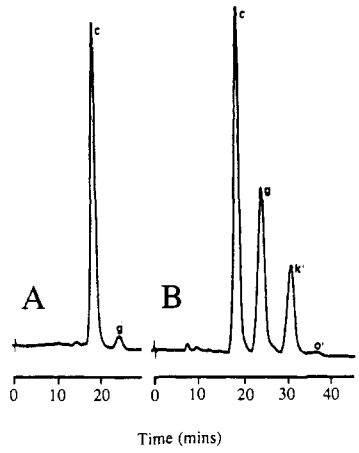


FIGURE 9: HPLC chromatograms of the natural mixture of Bmphs isolated from *C. vibrioforme* strain Bl-20 (A) in 1983 and (B) in 1984. Peak assignments: (c) [4-Et,5-Et,δ-H]; (g) [4-*n*-Pr,5-Et,δ-H]; (k) [4-*i*-Bu,5-Et,δ-H]; (o) [4-neoPn,5-Et,δ-H]. Pigments have *R* absolute stereochemistry at 2a unless primed (e.g., k') to indicate *S* absolute stereochemistry. HPLC conditions: Waters Associates μBondapak reversed-phase cartridge, Z-module, 2.5 mL/min of 85:15 methanol/water; Perkin-Elmer LC55B variable-wavelength detector set at 660 nm.

concomitant red shifts of the in vivo antenna pigment array. A new BChl-d producing organism was investigated in 1983; this *C. vibrioforme* strain Bl-20 had a long-wavelength in vivo absorption maximum at only 714 nm, compared with 725–736 nm usually observed in BChl-d producing strains (Smith & Goff, 1985). Figure 9A shows the HPLC profile of the Bl-20 Bmph-d after harvesting in early 1983 and compares it with a similar tracing after harvesting of another batch of the continuously grown Bl-20 strain in early 1984 (Figure 9B). The in vivo absorption maximum of the Bl-20 strain in 1984 had shifted from 714 to 728 nm, the latter being as expected for a normal BChl-d producing strain. Within the period of 1 year a strain which was producing almost entirely the [4-Et,5-Et]-BChl-d changed such that it was producing all BChl-d homologues, including the [4-neoPn,5-Et] compound. Interestingly, the relative amount of BChl-d homologues decreases with increasing numbers of methyl units (unlike the normal pattern), indicating that the Bl-20 strain was still in the process of alteration in 1984. In summary, BChl-d producing strains of bacteria experience (under current growth conditions in Davis) increased methylation at the 4- and 5-positions, reaching a stage at which the [4-*i*-Bu,5-Et]-BChl-d is the major component; absorption maxima in living cells concomitantly red shift from 714 to about 728 nm. A switch to δ-meso methylation then ensues, and the 4- and 5-alkylation process is reversed to a certain degree. In the final stage of the alteration process [4-Et,5-Et]-BChl-c is the major pigment being produced. Thus, a strain previously absorbing at 714 nm (containing almost completely [4-Et,5-Et]-BChl-d) shows an in vivo absorption maximum in the intermediate range 728–736 nm (with [4-*i*-Bu,5-Et]-BChl-d as the major pigment) and in the final stage a long wavelength of about 750 nm ([4-Et,5-Et]-BChl-c as the major pigment).

In the course of identification of the nature of the observed bathochromic shifts, in vitro studies with BChl-c **2** and BChl-d **1** (Table I) in a variety of organic solvents were carried out (Smith et al., 1983c) (Table II). The BChl-c show two long-wavelength absorption maxima, at 710 (shoulder) and 748 nm, in hexane (748 nm in vivo), whereas the BChl-d absorb at 728 nm (714–736 nm in vivo). Upon titration with coordinating solvents such as methanol, a hypsochromic shift of 60–90 nm occurred to give monomeric BChl, presumably

Table II: Electronic Absorption Maxima (Nanometers) for BChls from Green and Brown Bacteria

BChl	hexane	cyclohexane	hexane/MeOH[a]	CCl_4	$CHCl_3$
BChl-c (**2**)	430	432	430	434	432
	452	674	662	670	658
	(710)[c]	706	704		
	748				
BChl-d (**1**)	426	428	426	428	426
	442	662	656	656	658[b]
	728	694		(700)[c]	
BChl-e (**5**)	448	454	468	460	464
	520	662	600	660	658
	708	682	648	(700)[c]	

[a] Approximately 1% methanol in hexane was used for disaggregation of the oligomer. [b] Appears at 672 nm in carefully purified $CDCl_3$. [c] Parentheses indicate a shoulder.

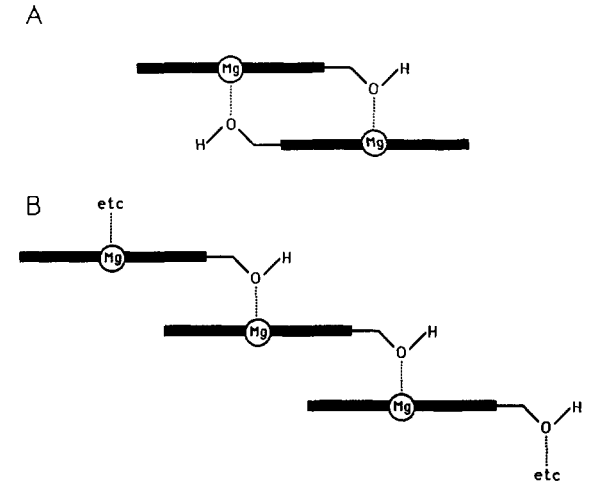


FIGURE 10: Major interactions responsible for aggregate formation in BChl-c, -d, and -e: (A) dimers; (B) higher oligomers. In (B) the opportunity is also present for the isocyclic ring carbonyl to coordinate with the magnesium in the BChl above.

with methanol coordinated to the central magnesium (Smith et al., 1983c). ESR studies of the oxidized BChl-c antenna of *Chlfl* (Betti et al., 1982; Smith et al., 1983c) suggested that about 14 BChl-c molecules are aggregated in this complex. Hence, although there is no evidence to suggest that the relationship between aggregate size and red shift is linear, it is reasonable to assume that aggregation of BChl-c in hexane involves 12–14 molecules as well, since the optical spectra in hexane and in living cells are virtually superimposable. Twelve to 14 BChl are believed to be associated with the 15-kDa protein subunits that comprise the antenna rod elements of Chlorobiaceae (Olson, 1980). Use of pure $CDCl_3$ as solvent leads to formation of an aggregate absorbing at 672 nm which has been definitively shown (Abraham et al., 1985; Smith et al., 1986) by proton NMR spectroscopy to be a dimer which is long-lived on the NMR time scale; the structure of the dimer was established by using a double-dipole ring current model (Abraham & Smith, 1983), and it was determined that each hydroxyethyl coordinated to the magnesium of the neighboring molecule (Figure 10A). Addition of methanol (or pyridine) to the $CDCl_3$ solution caused a hypsochromic shift to 656 nm and produced the monomeric species.

Extensive spectroscopic work on the aggregation of various Chls has been reported in the literature (Katz & Brown, 1983; Smith et al., 1986; Olson et al., 1986; Abraham et al., 1988). The structure of the BChl-c and BChl-d oligomeric species present in hexane has also been proposed to involve magnesium to hydroxyethyl interactions (Smith et al., 1983c) (Figure

Table III: Long-Wavelength Maxima for BChl-d (1) Monomer and Aggregates

species	λ_{max} (nm)	$\lambda_{Olig}-\lambda_{monomer}$ (nm)	solvent
monomer	656		CH_2Cl_2/MeOH
dimer	672	16	$CDCl_3$
12–14 oligomer	730	74	hexane

10B), and a significant body of evidence (Brune et al., 1988) has been published in support of this model which can be disaggregated to monomer by addition of methanol; it should also be mentioned, however, that some resonance Raman work (Lutz & van Brakel., 1988) apparently does not support the model. Thus, it appears that the magnitude of the red shift in BChl-d can be correlated, at least in vitro, with the molecular size of the aggregate (Table III), though not necessarily in a linear fashion.

It has been shown (Smith & Goff, 1985) that the size of the red shift can also be correlated with the nature of the substituents present at the 4-position in the Bmph-d (Figure 9). When R^4 = Et, the antenna system in living cells (e.g., *C. vibrioforme* strain B1-20) absorbs at 714 nm, but with R^4 = Et, *n*-Pr, *i*-Bu, or neoPn (in the same strain after growth under reduced light conditions) the antenna system absorbs at 728 nm (Smith & Goff, 1985). Eventually, as discussed above, the δ-meso position in the BChl-d is methylated (Smith & Bobe, 1987), introducing an additional 20-nm red shift (from ca. 728 to ca. 748 nm), and the BChl-c are produced.

We interpret the gradually increased alkylation at the 4 side chains (Figures 1–3), even in subcultures derived from single cells, to be a consequence of dim light cultivation. Whether selection of a mutant, or adaptation, or a combination of both is responsible for the observed phenomena is not clear at this time. Since the proportions of 4-Et, 4-*n*-Pr, and 4-*i*-Bu homologues have been shown to vary with light conditions (Huster & Smith, 1989), the overall process seems adaptive; this is not to say, however, that dim light did not provide conditions under which mutants became selected. Others have shown (Broch-Due & Ormerod, 1978) that cultivation of *Chlorobium limicola* forma *thiosulfatophilum* (a BChl-d producer) under low light intensity conditions caused a red shift in living cells of more than 10 nm, and this was interpreted as a selection of a BChl-c producing mutant.

The batch culture conditions that were employed are likely to cause changes of the kind we have observed. Plants, when subjected to reduced light conditions, can respond by producing more chlorophyll, but at steady state, though reshuffling of cell components is possible, the bacteria are unable to produce more BChl because they lack the necessary nutrients. In the exponential growth phase the increasing cell density must result in increasing self-shading of the cells. During the stationary growth phase, nutrients may become available from lysed cells. Cells developing under these conditions (Huster & Smith, 1990) may synthesize BChl antenna arrays more suited for photosynthesis under reduced light conditions; this may be accomplished either by absorbing at a wavelength different from that of the existing cells or by red shifting and thereby absorbing radiation of longer wavelength which penetrates deeper into the culture.

The structural basis for the red shift is presumably one which increases the size of the antenna aggregate (Smith et al., 1983c; Brune et al., 1988) in living cells by loading more BChls onto the antenna rod protein template; the 5.6-kDa BChl-c binding polypeptide in the rod-shaped chlorosome elements of *Chlfl* has been sequenced, and possible BChl-c binding sites have been proposed (Wechler et al., 1985). Production of more highly alkylated BChls produces this effect (Figure 9) possibly by increasing hydrophobic interactions in the antenna stack. Alternatively, and we prefer to suggest, the increased aggregate size (and increased red shift) may be related to a change in the chirality (Table I) at the 2a-position from *R* to *S* as R^4 changes from Et to *i*-Bu (Smith et al., 1983a; Smith & Goff, 1985). Conversion of the 2-vinyl to afford 2-(1-hydroxyethyl) (or possibly reduction of a 2-acetyl) in a biosynthetic precursor is very likely a late step in the biosynthetic pathway, which takes place after the homologation/methylation steps have been accomplished; thus, we suggest that it is the size of the 4- and 5-alkyl groups which regulate the chirality of the 2-(1-hydroxyethyl), rather than vice versa. The proportions of *R* and *S* diastereomers in the natural pigment mixture (e.g., 100% *R* for 4-Et,5-Et-Bmph-d to 100% *S* for 4-neoPn,5-Et-Bmph-d) argue against racemization or even epimerization at some step in the biosynthetic pathway. Thus, appearance of new BChl pigments in the medium which possess higher homologation at positions 4 and 5 must be related to de novo synthesis, rather than simple homologation or modification of existing BChl-c or -d molecules. Note also that the hydroxyethyl is almost certainly involved in the major interaction (Figure 10B) responsible for holding the aggregate together (ignoring protein contacts). Figure 11 shows how chirality at position 2a might stabilize (Figure 11A) or destabilize (Figure 11B) a developing antenna aggregate, once the protein has introduced the primary stereochemistry of the array. In hexane (i.e., in the absence of protein) pure 2a-*R* and 2a-*S* homologues give the same magnitude of red shift, as do R^4 = Et and R^4 = *i*-Bu (Smith et al., 1983c). Thus (Figure 11), small R^4 substituents will tend to destabilize and discourage large aggregate formation by indirectly influencing the stereochemistry at the 2a carbon, and as R^4 is increased in size, larger aggregates should statistically result because of the more favorable stereochemical situation about the coordinating site (Figure 11A). The phenomena exhibited by green bacteria in response to decreased light availability are discussed further in the accompanying paper (Huster & Smith, 1990).

An alternative possibility for the red shift could be buckling of the macrocycle due to the presence of the additional carbon homologation, and that is certainly a possibility in the case of the δ-meso-methyl substituent which would be expected to interfere sterically with the abutting 1- and 8-substituents; in organic solvents such as ether, the BChl-d absorb at 650 nm while the BChl-c have their long-wavelength maximum at 660 nm (hence giving rise to the original nomenclature of the BChl-d and -c as the *Chlorobium* Chls 650 and 660, respectively). Indeed, in living cells there exists an approximately 20-nm difference in the long-wavelength maxima (730 vs 750 nm, vide supra). Unfortunately, this possible buckling phenomenon cannot be quantified; there appears to be no difference in NMR ring current effects between the Bmph-c and Bmph-d (Smith et al., 1983a; Smith & Goff, 1985), and though numerous X-ray structures of Bmph-d homologues indicate a planar macrocycle with only minor perturbation in the reduced ring (Smith et al., 1982, 1983b), no X-ray structure of a Bmph-c has yet been reported. On the other hand, we consider it to be very unlikely that homologation of 4-Et to 4-*n*-Pr (or to *i*-Bu and neoPn) would cause any significant buckling of the macrocycle plane because the 4-position is much less sterically constrained than a meso position and because the alkylation is taking place two carbons away from the macrocycle. This conclusion is reinforced by the fact that the optical spectra in organic solvents of the Bmph-d

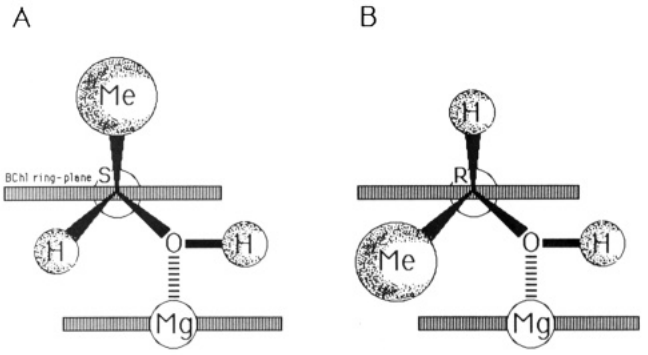


FIGURE 11: Diagrammatic representation of 2-(1-hydroxyethyl)-to-magnesium interaction in (A) 2a-(*S*)-BChl and (B) 2a-(*R*)-BChl (possibly destabilized by interplanar steric congestion with the 2a-methyl). The view is along the 2a–2 bond.

bearing a 4-Et is not blue-shifted compared with that of the corresponding 4-neoPn Bmph-d (cf. the 10-nm shift for the corresponding Bmph-d to Bmph-c 4-Et pair of pigments), and X-ray structures of the 4-Et,5-Et-Bmph-d (Smith et al., 1982) and the 4-neoPn,5-Et-Bmph-d (Smith et al., 1983b) show similar planarity of the chromophore.

We (Figure 8) and others (Brune et al. 1987) have also observed that the *Chlfl* J-10-fl BChl-c contain traces of BChl-d. Our suggestion is that [4-Et,5-Me]-BChl-d once was the predominant pigment in *Chlfl* and that it has become meso methylated with time to accommodate external environmental conditions. One might also make similar conclusions about the BChl-*e* (**5**) from *Chlorobium phaeovibrioides*, which also are δ-meso methylated (Simpson & Smith, 1988); the name BChl-f has been assigned to the corresponding δ-meso-H compounds (**6**), presumably in anticipation that they may one day be discovered. However, the observed presence of very minor amounts of (presumed) BChl-f in the BChl-e culture, along with the complete absence of any 5-Me homologues (Simpson & Smith, 1988), suggests to us that this strain has already changed to its meso-methylated form.

Conclusions

Experimental data gathered thus far point to a light adaptation process in which the lipophilicity (i.e., number of methyl units attached to the 4-, 5-, or δ-meso-positions) of the macrocyclic periphery determine the extent of aggregation in the antenna system and therefore its size and wavelength of absorption maxima. Hydrophobic interactions between individual BChl molecules may become stronger with increasing number of methyl units, giving rise to larger aggregates. Alternatively, the additional methyl units attached to the 4-position shift the absolute configuration of the 2-(1-hydroxyethyl) group from pure *R* in the case of 4-Et to pure *S* in the case of 4-neoPn, which in turn might determine the size of the in vivo aggregates due to the intrinsic nature of the pigment protein system. Thus, larger aggregates [with 2-(*S*)-hydroxyethyl] tend to absorb light at about 750 nm, whereas smaller aggregates [with 2-(*R*)-hydroxyethyl] experience a hypsochromic shift of as much as 36 nm (strain B1-20; R^4 = Et; 714 nm) depending upon their size.

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Biosynthetic Studies of Substituent Homologation in Bacteriochlorophylls *c* and *d*†

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ABSTRACT: Administration of carbon-13 and carbon-14 labeled glutamate, glycine, and methionine to *Chlorobium vibrioforme* forma *thiosulfatophilum* strain D have demonstrated operation of the C_5 and C_1 metabolic pathways in bacteriochlorophyll *c* and bacteriochlorophyll *d* biosynthesis in this organism, with glutamate providing the δ-aminolevulinic acid for macrocycle synthesis and glycine providing the source of the extra homologation at the 4-, 5-, and δ-positions (via *S*-adenosylmethionine). Further evidence showing that the bacteria appear to adjust the homologue composition of their antenna bacteriochlorophylls in response to varying growth conditions is presented. Timing of these changes within a single culture is consistent with a light adaptation mechanism, which predicts that degree of alkylation is directly proportional to light intensity in the culture; other factors influencing pigment composition during the lifespan of a single culture may also be operating, and these are discussed.

Bacteriochlorophylls *c* (BChl-c,[1] **1**) and *d* (BChl-d, **2**) are found in strains of green sulfur bacteria (Pfenning, 1977; Allen, 1966; Holt, 1965). They occur as homologous mixtures (Table I; **1a–f**; **2a–h**), and their complete structural features have been established (Smith et al., 1983a; Smith & Goff, 1985). Both BChl-c and -d contain components with extra methyl substituents (relative to Chl-a) in the 4 and 5 side chains, and the BChl-c possess a δ-meso methyl group, which is responsible for a 20-nm red shift of the long-wavelength absorbance band in living cells (at ca. 750 nm compared with ca. 730 nm for the BChl-d) (Olson, 1980; Gloe et al., 1975). Feeding experiments with ^{13}C-enriched methionine have shown (Kenner et al., 1978) that these extra methyls are derived from the *S*-methyl of methionine, via *S*-adenosylmethionine (SAM).

The organism used in most of our previous studies was the anaerobic green sulfur bacterium *Chlorobium vibrioforme* forma *thiosulfatophilum* strain NCIB 8327, referred to hereafter as *Chlb* 8327. For at least 20 years, since Holt and co-workers first reported the isolation and characterization of Chls from *Chlb* (Holt & Hughes, 1961; Hughes & Holt, 1962; Holt & Purdie, 1965), this strain appears to have produced almost entirely the non-meso-methylated BChls-d. But in the period from 1980 to 1984, changes were observed (Smith & Bobe, 1987; Bobe et al., 1990) in the pigment composition toward more meso-methylated species (currently the ratio of BChl-c:BChl-d in this organism ranges from 2:1 to 10:1, depending on the age of the particular culture), as well as increased alkylation at the 4 and 5 side chains. Once the possibility of contamination in the culture by BChl-c producing species was eliminated, it was proposed (Bobe et al., 1990; Smith & Bobe, 1987) that the bacterium itself had undergone an adaptation to shift the absorption maximum of its antenna system to longer wavelength in response to reduced availability of light. The UC Davis adapted strain of *Chlb* 8327 was named *Chlb* strain D. Using spectrophotometry, other workers had also shown (Broch-Due & Ormerod, 1978) that cultivation under low light intensity conditions of *Chlorobium limicola* forma *thiosulfatophilum* (a BChl-d producer) caused a red shift in the antenna array of more than 10 mm, and this was interpreted as a selection of BChl-c producing mutants. It is readily apparent that meso methylation results in a 20-nm red shift in vivo, but the driving force behind increased 4- and 5-alkylation was not so obvious until it was determined (Smith & Goff, 1985) that bacterial strains absorbing at relatively short wavelength (e.g., *C. vibrioforme* strain B1-20, λ_{max} 714 nm) possess largely R^4 = Et, while the normal strains of *Chlb* featuring the full range of R^4 substituents in their mixture of antenna pigments (i.e., R^4 = Et, *n*-Pr, *i*-Bu, neoPn) absorb at ca. 730 nm. It may be that an increase in lipophilicity of the macrocyclic periphery increases the degree of aggregation in the BChl antenna array (thereby causing a red shift) or else that alteration of the absolute stereochemistry at the 2-(1-hydroxyethyl) from *R* (for R^4 = Et) to *S* (for R^4 = *i*-Bu) affects aggregate size because one of the major intermolecular interactions in the antenna array involves coordination of the oxygen in the chiral hydroxyethyl with a neighboring magnesium atom (Smith et al., 1983b). It has been shown in vitro that the magnitude of red shift from monomer to aggregate is related to the size of the aggregate in organic solvents (Smith et al., 1983b, 1986; Abraham et al., 1985).

Another type of organism used briefly in our studies is the green sulfur bacterium *Prosthecochloris aestuarii* strain SK

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[1] Abbreviations: BChl, bacteriochlorophyll; Chl, chlorophyll; *Chlb* 8327, *Chlorobium vibrioforme* forma *thiosulfatophilum* strain NCIB 8327; *Chlb* strain D, a *C. vibrioforme* forma *thiosulfatophilum* strain, derived from *Chlb* 8327, which now produces substantially the BChl-c instead of the BChl-d; SAM, *S*-adenosylmethionine; *n*-Pr, *n*-propyl; *i*-Bu, isobutyl; neoPn, neopentyl; ALA, δ-aminolevulinic acid; Bmph, methyl bacteriopheophorbide; NMR, nuclear magnetic resonance; TLC, thin- or thick-layer chromatography; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran.

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