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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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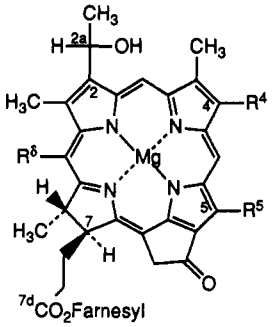
Table I: Structural Assignments for the BChl-c and BChl-d



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

[a] The corresponding methyl bacteriopheophorbides (Bmphs) are transesterified with methanol (instead of farnesol) at the 7d ester and have the magnesium removed.

413 (combined with the nonphotosynthetic organism *Desulfuromonas acetoxidans* strain 5071). *P. aestuarii* produces almost entirely BChl-c antenna pigments, although the presence of minor amounts of BChls-d has been noted in the C. e. (formerly *Chloropseudomonas ethylica*) strain (Smith et al., 1983a), in the SK 413 strain, and in other mainly BChl-c producers (Richards & Rapoport, 1967). Interestingly, the BChls from *P. aestuarii* show a reduced degree of R^4 alkylation, with the 4-Et homologue accounting for >70% of the product mixture (Smith et al., 1980; Holt, 1965). This is in comparison with the BChls-c from *Chlb* strain D, where the ratio of Et:*n*-Pr:*i*-Bu R^4 substituents is closer to 1:2:1 (Bobe et al., 1990; Smith & Bobe, 1987). *P. aestuarii* pigments achieve a 20-nm red shift by a single meso alkylation and may therefore not need the somewhat smaller shifts which can be achieved by modification of R^4. *Chlb* strain D, on the other hand, is still in the process of switching from a BChl-d to a BChl-c producer, and so it still relies somewhat on increased R^4 alkylation to red shift its antenna system.

Experimental Procedures

General. Electronic absorption spectra were measured on a Hewlett-Packard 8450A spectrophotometer using solutions in dichloromethane. ^{13}C NMR spectra were obtained in $CDCl_3$ at 75 MHz and proton NMR spectra at 300 MHz (GE QE300) with chemical shifts reported in parts per million relative to internal $CDCl_3$. Reactions were monitored by using thin-layer chromatography (TLC) using commercially available Eastman-Kodak 13181 (100 μm thick) silica sheets. Gravity and flash column chromatography employed either Merck neutral alumina (70–230 mesh) or Merck silica gel 60. The alumina was usually deactivated with 6% water (Brockmann grade III) before use. Preparative thick-layer chromatography was carried out on 20 × 20 cm glass plates coated with Merck G 254 silica gel (1 mm thick). [2-^{13}C]Glycine (99 atom %) was obtained from ICN Stable Isotopes. [1-^{13}C]-*dl*-Glutamic acid (99 atom %) and [^{13}C-CH_3]methionine (98.7 atom %) were obtained from MSD Isotopes. [1-^{14}C]-*l*-Glutamic acid (0.05 mCi, 0.15 mg) and [2-^{14}C]glycine (0.05 mCi, 0.075 mg) were obtained from New England Nuclear. [^{14}C-CH_3]Methionine (5.28 mCi/mol) was prepared from $^{14}CH_3I$ and homocysteine (Kenner et al., 1978).

Photosynthetic Bacteria Culture Techniques (Beeftink, 1983). The medium [composition as previously described (Smith & Goff, 1985)] was bubbled briskly with CO_2 gas for about 5 min, to purge the flask of oxygen and to lower the pH. While bubbling, the flask was inoculated with mother culture (volume ratio $^1/_3$ to $^1/_{10}$) and then sealed with a rubber stopper. The 1- or 2-L Erlenmeyer flasks were placed on a magnetic stirrer approximately 50 cm from a bank of six 40-W 48-in. soft white fluorescent light tubes. Growth at 30 (±2) °C was usually apparent within 2–3 days. No attempts were made to maintain strict sterile conditions during the subculturing procedure.

Extraction of Bmphs. Cells were harvested by filtration on a bed of Celite. Alternatively, cells could be centrifuged at 5000*g* in 250-mL centrifuge bottles and the cell pellet slurried with a little water and adsorbed on Celite. The Celite–cell paste was frozen until extracted. Pigments were extracted from the cell–Celite mixture with 2:1 acetone–diethyl ether. Usually one extraction, with thorough rinsing of the Celite with solvent, was sufficient to remove >90% of the pigments. The organic solvents were evaporated, and the pigments were partitioned into CH_2Cl_2, washed twice with brine, dried over $MgSO_4$, and then evaporated. The BChls were transesterified in 3% (v/v) H_2SO_4 in methanol for 3 h at room temperature. The esterification reaction mixture was poured into CH_2Cl_2, washed three times with brine, dried over $MgSO_4$, and evaporated to give a residue consisting of Bmphs and carotenoids.

The Bmphs were purified on a neutral alumina (Brockmann grade III) gravity column. Carotenoids were eluted with 4:1 hexane–CH_2Cl_2 followed by 1:1 hexane–CH_2Cl_2. Other colored side products were removed by elution with 100% CH_2Cl_2. Finally, elution with 1% methanol in CH_2Cl_2 gave the Bmphs as a narrow brown-black band. The product obtained consisted of both the 5-ethyl and 5-methyl homologues. Separation of these homologues (before HPLC) was achieved with preparative silica gel TLC, eluting with 95:5 CH_2Cl_2–THF. Usually one development was sufficient to separate the 5-ethyl (R_f = 0.46) from the 5-methyl (R_f = 0.38) homologue. If it was known beforehand that the individual homologues must be separated, the alumina column was eliminated and the entire separation of the Bmphs from the 2-vinyl side product (R_f = 0.62) and carotenoids (R_f = 0.75 and 0.91) was accomplished on preparative silica gel TLC.

Microscale Method for Bmph Extraction. This method uses all disposable equipment for extraction and purification of the Bmphs and is ideal for small amounts of radiolabeled material. The collected cell pellet was treated in a 16 × 125 mm culture tube with 2 mL of acetone for 0.5 h with occasional shaking. A medium spatula full of Celite was added, followed by 1 mL of diethyl ether, and the solution was mixed well and allowed to stand another 10 min. The Celite was pelleted by centrifugation in a table-top swinging-bucket centrifuge, and the clear green supernatant, containing BChls and carotenoids, was poured into a 13 × 100 mm culture tube. The pigment solution was evaporated under a stream of N_2 to <0.5 mL volume, and the residue was dissolved by addition of 1 mL of methanol. The methanol solution, along with any undissolved

solids, was transferred to a 1.5-mL Eppendorf centrifuge tube. The solution was again evaporated under an N_2 stream to <0.5 mL and then evaporated to dryness in a Speed-Vac heated vacuum centrifuge. The residue was transesterified with 1.0 mL of 3% (v/v) H_2SO_4 in methanol for 3 h at room temperature. The reaction mixture was neutralized with 0.56 mL of 2 N methanolic KOH, which resulted in salt precipitation.

The methanol–salt slurry was treated with 4.8 mL of water to dissolve the salts. This suspension (about 1:3 methanol–water) was passed through a C_{18} SepPak cartridge (Waters Associates), which had previously been wetted with methanol and equilibrated with 1:3 methanol–water. The SepPak cartridge was washed with 3 mL of water and then 12 mL of 1:3 methanol–water. Bmphs were eluted with 3 mL of 19:1 methanol–water. The nonplar carotenoids were retained by the cartridge and effectively separated from the Bmphs. This method, however, does not separate the 5-methyl/5-ethyl homologues or the 2-vinyl dehydration product.

Feeding Studies with ^{13}C-Labeled Amino Acids. For the [^{13}C]glycine and [^{13}C]glutamate feedings, three 2-L subcultures of *Chlb* strain D (Smith & Bobe, 1987; Bobe et al., 1990) were prepared (two experimental, one control) and fed at the time of subculturing with enough amino acid to give an 0.5 mM final concentration. These cultures were grown for 8 days and harvested and the Bmphs were extracted and purified on a neutral alumina gravity column. Yield of Bmph was 20 mg (glutamate), 24 mg (glycine), and 32 mg (control). After ^{13}C NMR spectra were obtained, the homologous mixtures were separated by silica gel preparative TLC and C_{18} reversed-phase preparative HPLC (Dynamax column, 85:15 MeOH–H_2O, 20 mL/min flow rate) into their individual homologues. For the glutamate and control feedings, only the [4-*n*-Pr,5-Et]-Bmphs-c and -d were isolated; for the glycine feeding, the [4-Et,5-Me]-, [*n*-Pr,Me]-, and [*i*-Bu,Me]-Bmphs-d and the [Et,Et]-, [*n*-Pr,Et]-, and [*i*-Bu,Et]-Bmphs-c and -d were isolated. The separated homologue solutions from preparative HPLC were concentrated under vacuum and partitioned into CH_2Cl_2 and brine. The separated organic solutions were dried over $MgSO_4$, filtered, and evaporated, followed by ^{13}C NMR analysis of the residues.

NMR (75 MHz, $CDCl_3$): (A) alkyl substituents 30.76 [4-$CH_2C(CH_3)_3$], 23.21 [4-$CH_2CH(CH_3)_2$], 20.47 (δ-meso CH_3), 16.82 (5-CH_2CH_3), 14.46 (4-$CH_2CH_2CH_3$); (B) macrocycle Bmph-d [*n*-Pr, Et], 160.1 (7′), 151.4 (4′), 149.3 (6′), 135.1 (2′), 106.1 (γ), 104.5 (β), 97.7 (α), 92.6 (δ); Bmph-c [*n*-Pr,Et] 158.7 (7′), 151.2 (4′), 147.6 (6′), 132.8 (2′), 105.9 (γ,δ), 102.9 (β), 97.5 (α).

[^{13}C]Glycine Pulsed Feeding Experiments. Five 1-L subcultures of *Chlb* strain D were inoculated all at the same time. At times t = 0, 7, 14, 21, and 28 days one each of the subcultures was fed with 1.0 mL of a stock solution of [2-^{13}C]-glycine, 0.5 M (final glycine concentration in culture 0.5 mM). Five days after feeding, each culture was harvested and extracted, and the Bmph mixture was purified as above. Proton-decoupled ^{13}C NMR spectra were obtained by using the following parameters: pulse width, 4.5 ms (30° pulse); acquistion time, 409.6 ms; recycle time, 2.00 s.

Long-Term [^{13}C-CH_3]Methionine Feeding. One 1-L subculture of *Chlb* strain D was prepared as usual and allowed to grow for 38 days (well into the plateau phase of growth). At this time, the culture was fed with 164 mg of [^{13}C-CH_3]methionine (final concentration of methionine 1 mM) and allowed to stand in dim light for another 75 days. The Bmphs were isolated, and ^{13}C NMR spectra were obtained as above.

Feeding Studies with ^{14}C-Labeled Amino Acids. For the [^{14}C]glycine and [^{14}C]glutamate feedings, two 2-L subcultures were fed at the time of culturing with 0.05 mCi of radiolabeled amino acid plus enough cold amino acid to give a 0.5 mM final concentration. These were grown for 4 days, and the Bmph mixture was isolated as above. A small aliquot was removed (1% of the total) and bleached with chlorine gas, and the radioactivity was counted to determine percent incorporation. The remainder of the radiolabeled Bmphs were separated into their individual homologues by using preparative TLC and analytical HPLC (C_{18}, 7.8 × 300 mm steel column, 85:15 MeOH–H_2O, 2.0 mL/min flow rate). The HPLC column effluent was collected in 1-min fractions and counted, without bleaching, to determine radioactivity. Summing the background-corrected counts under each peak gave the "radioactive integration", and this was divided by the A_{405} integrated peak area to give the specific activity for each homologue.

Radioactive Scintillation Counting. Samples for radioactive counting were placed in scintillation vials and bleached under a slow stream of Cl_2 gas for about 30 s, followed by flushing with N_2 for 10 min to remove excess chlorine gas. Scintillation cocktail (Aquasol 2) was added, and the vials were shaken well and placed in the dark for 1–2 h before counting. A vial containing only scintillation cocktail was similarly counted to determine background. After cpm for the sample and background were measured, a measured amount of standard [^{14}C]hexadecane was added to both the sample and background vials, which were counted again to calculate percent counting efficiency (%CE) and disintegrations per minute (dpm):

$$\%CE = \frac{\text{cpm(sample + std)} - \text{cpm(sample)}}{\text{cpm(std)} - \text{cpm(background)}} \times 100$$

$$\text{dpm} = [\text{cpm}/\%CE] \times 100$$

Radioactive BChl Turnover Experiment. Two 250-mL subcultures were prepared with *Chlb* strain D (Smith & Bobe, 1987), variety 3 (almost entirely a BChl-c producer) and variety original (BChl-d 10–30%). These were fed at the time of subculturing with ca. 14 mg (0.47 mCi) each of [^{14}C-CH_3]methionine. After 38 days of growth (well into the plateau phase) a 30-mL aliquot was removed from each culture, and the cultures were placed in dim light. Cells were collected from the aliquots by centrifugation in a table-top swinging-bucket centrifuge for 20 min, using two disposable 16 × 125 mm culture tubes (15 mL per tube). The clear supernatant growth medium was poured off, the test tube was covered with Parafilm, and the cell pellet was frozen until Bmph extraction. Aliquots were removed again at 113 and 228 days, and the cells were collected and frozen as described. Bmphs were extracted from the cell pellets according to the microscale method described above. The 3 mL of Bmph solution eluted from the SepPak cartridge was diluted to 5 mL with methanol. Of this, three 1.3-mL portions were placed in three scintillation vials, bleached with chlorine gas, and counted as described above to obtain the activity of each sample. Of the remainder, three 0.25-mL portions were placed in separate 13 × 100 mm culture tubes and diluted 10-fold with methanol, and the absorbance at the Soret band (A_{Soret}) was measured to obtain the relative amount of Bmph in each sample. Specific activity was then calculated as dpm/(A_{Soret}).

Results and Discussion

Feeding Studies with [^{13}C]- and [^{14}C]Glutamate and [^{13}C]- and [^{14}C]Glycine. The biosynthesis of the chlorophyll precursor ALA from glutamate via the C_5 pathway has been well

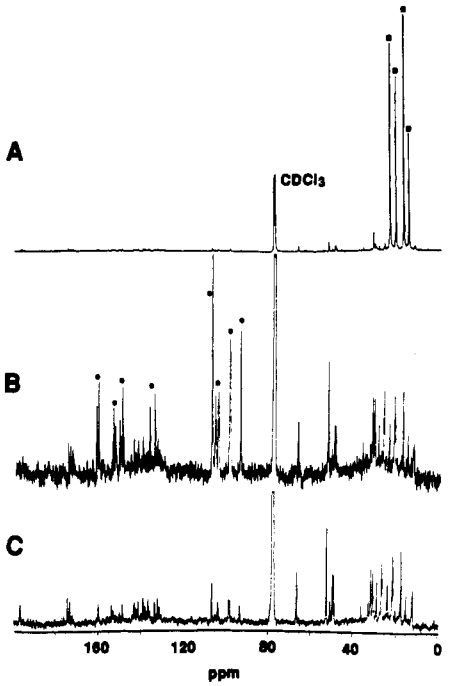


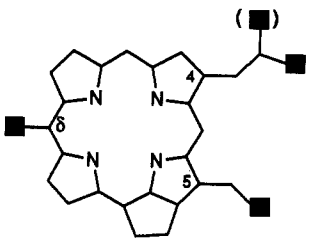
FIGURE 1: 75-MHz ^{13}C NMR spectra of homologous mixtures of Bmphs obtained from *Chlb* strain D grown (C) without any ^{13}C source, (B) with 99% enriched D,L-(1-^{13}C)-glutamate; and (A) with 99% enriched [2-^{13}C]glycine.

established in studies with green plants, algae, and most non-purple photosynthetic bacteria, although some exceptions have been noted (e.g., *Scenedesmus obliquus*, potato tuber), and many photosynthetic organisms use the Shemin pathway (glycine and succinate) for mitochondrial and cytosolic ALA synthesis (Castelfranco & Beale, 1983; Rebeiz & Lascelles, 1982; Hoober, 1987). Because of reports from Oh-Hama and co-workers, who studied glycine and glutamate incorporation into BChl-a in *Chromatium vinosum* (Oh-Hama et al., 1986a), we were prompted to investigate the metabolic pathway of chloroplast ALA in our Davis strain (strain D) of *Chlb*. Monitoring ALA formation from glycine or glutamate in a number of organisms has shown (Andersen et al., 1983) that *Chlorobium* species use glutamate almost exclusively for their ALA production.

The *Chlb* strain D was cultured in the normal way, in the presence of either L-[1-^{14}C]glutamic acid or [2-^{14}C]glycine (0.05 mCi/L), with cold amino acid added to a final concentration of 0.5 mM. After incubation for 4 days, cells were harvested, the methyl bacteriopheophorbides (Bmphs) were isolated, and a small aliquot (1% of the total yield) was taken for radioactive counting. Surprisingly, it was found that both amino acids were utilized, with glutamate and glycine giving incorporations of 1.6% and 0.6%, respectively. [Incorporation = 100(counts isolated/counts fed)%.] These initial carbon-14 feedings were not further optimized.

These preliminary incorporations indicated the possible operation of a dual pathway. Since both incorporations were siginificant, the feeding experiments were repeated with D,L-[1-^{13}C]glutamic acid and [2-^{13}C]glycine (99 atom %), incubating for 8 days before harvest. Figure 1C shows the natural abundance ^{13}C NMR spectrum of the Bmph mixture produced by this strain of bacteria. Figure 1B shows the spectrum from the ^{13}C-enriched glutamate feeding; on the basis of previously published ^{13}C assignments (Kenner et al., 1978; Smith et al., 1980; Oh-Hama et al., 1986b), it is clear that C_1 of glutamate is incorporated into ALA via the C_5 pathway. The observation of enrichment in more than four meso and four α carbon resonances is due to the presence of several Bmph homologues in the product mixture.

In contrast, the glycine feeding (Figure 1A) resulted in enrichment of only the meso methyl and the terminal carbons of the 4-*n*, 4-*i*-Bu, 4-neoPn, and 5-Et substituents (Kenner et al., 1978; Smith et al., 1980). In the expanded spectrum (not shown), no evidence for incorporation of the C_2 of glycine into the meso or quaternary macrocycle skeleton carbons was apparent. Since it has been established that these methyls are derived biosynthetically from methionine (Kenner et al., 1978), the results of our feeding studies clearly demonstrate that glycine is incorporated into methionine via the C_1 metabolic pool. Glycine C_2 metabolism through N^5-methyltetrahydrofolate is a widely observed process in numerous organisms (Cossins, 1980). Feeding studies by Oh-Hama and others with [2-^{13}C]glycine have shown enrichment of the methionine-derived 10b methoxy carbon of chlorophyll *a* in green algae (Oh-Hama et al., 1985b) and maize (Porra et al., 1983) and of BChl-a in *Rhodopseudomonas* (Oh-Hama et al., 1985a) and *Chromatium* (Oh-Hama et al., 1986a). However, Oh-Hama et al., in work published (Oh-Hama et al., 1986b) at about the same time as our preliminary publication relating to the present work (Smith & Huster, 1987), reported that the glycine-to-methionine transfer is *not* operative in the green sulfur bacterium *P. aestuarii*, on the basis of their observation of no ^{13}C enrichment in the BChl-c meso methyl and terminal R^4 and R^5 carbons after 20 h of incubation with [^{13}C]glycine. This directly contradicts our own findings, both in *Chlb* strain D (Smith & Huster, 1987) and in separate [^{13}C]glycine feeding experiments with our own cultures of *P. aestuarii* strain SK 413 (Huster, 1988), which clearly demonstrated incorporation of glycine into the methionine-derived "extra" carbons of BChls-c and -d from these bacteria (structure 3). These



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contradictory findings can probably be explained by our much longer incubation times with the labeled amino acid (8 days vs 20 h), which would allow time for the culture to grow and become more opaque (Bobe et al., 1990; Smith & Bobe, 1987), under which (self-shading) conditions the bacteria produce more highly alkylated BChl.

Quantitative Studies of Glutamate and Glycine Incorporation. Having in hand a considerable quantity of Bmph derived from administration of [^{13}C]- and [^{14}C]glutamate and [^{13}C]- and [^{14}C]glycine, we decided to quantitatively investigate the relative incorporation of these amino acids into the individual alkyl homologues of the Bmphs, separated by preparative TLC and HPLC. The Bmphs from the [1-^{13}C]-glutamate feeding were separated, and only the [4-*n*-Pr,5-Et] homologues of both the Bmphs-c and -d were isolated, since these represent the largest portion of the mixture. As expected, the enriched carbon-13 NMR resonances (not shown) were similar to those reported by Smith and co-workers for the α, β, γ, and δ meso and the 2′, 4′, 6′, and 7′ a-carbons in [4-*n*-Pr,5-Et] Bmph-c from *P. aestuarii* (Smith et al., 1980) and Bmph-d from *Chlb* 8327 (Smith & Goff, 1985). The discrepancy noted was an observed enrichment at 132.8 ppm

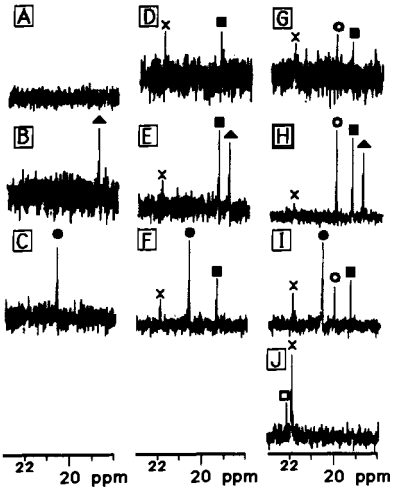

FIGURE 2: 75-MHz ^{13}C NMR spectra of Bmphs obtained from *Chlb* strain D grown with (2-^{13}C)glycine: (A) Bmph-d [4-Et,5-Me]; (B) Bmph-d [4-*n*-Pr,5-Me]; (C) Bmph-d [4-*i*-Bu,5-Me]; (D) Bmph-d [4-Et,5-Et]; (E) Bmph-d [4-*n*-Pr,5-Et]; (F) Bmph-d [4-*i*-Bu,5-Et]; (G) Bmph-c [4-Et,5-Et]; (H) Bmph-c [4-*n*-Pr,5-Et]; (I) Bmph-c [4-*i*-Bu,5-Et]; (J) Bmph-c [4-neoPn,5-Et]. Assignments: ○, δ-Me; ■, 5-Et; △, *n*-Pr; ●, *i*-Bu; □, neoPn; x, impurity.

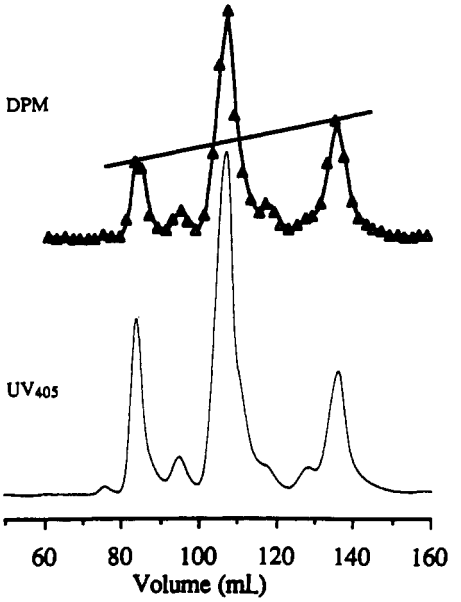

FIGURE 3: C-18 reversed-phase HPLC traces of 5-Et-Bmphs isolated from *Chlb* strain D grown with [^{14}C]glycine.

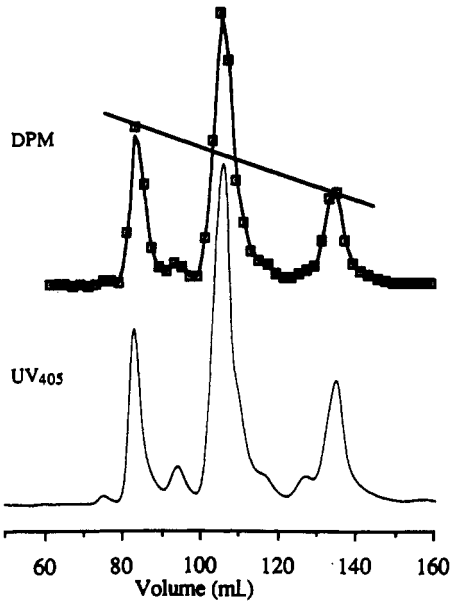

FIGURE 4: C-18 reversed-phase HPLC traces of 5-Et-Bmphs isolated from *Chlb* strain D grown with [^{14}C]glutamate.

which was assigned to the 2′ carbon, as opposed to the tentative assignment of 140.3 ppm for this position (Smith et al., 1980) and Goff's more recent reassignment of 137.9 ppm (Goff, 1984).

Of greater significance, however, are the ^{13}C NMR results from the separated homologues of the [^{13}C]glycine-derived Bmphs (Figure 2). It can be seen that, within the quantitative limits of error in the NMR experiment, the peak heights of the enriched resonances are in proportion to the occurrence of each methionine-derived methyl in the individual homologue. In other words, the peak heights of the terminal 4-*n*-Pr, 5-Et, and δ-meso methyl resonances are approximately equal, while the height of the terminal 4-*i*-Bu peak (representing contribution from two methyls) is roughly double that of the other peaks (Figure 2F,I). This is a significant observation, because it indicates that the ^{13}C-enriched methyls were incorporated into the Bmphs at about the same time, from a methionine pool of relatively consistent enrichment, assuming that the equilibration time of the label is shorter than the growth period. Thus, a single ^{13}C feeding, either at the time of subculturing or anytime during the culture's growth curve (i.e., a "pulsed" feeding), should provide a fairly accurate "snapshot" of relative [^{13}C]methyl incorporation into the alkyl and meso substituents, enabling one to approximate the relative amounts of the various homologues being produced at a particular point in the growth curve, particularly when a ubiquitous substrate such as glycine has been administered to the system. If this were not the case, i.e., if the 5b-methyl was incorporated at day 2 and the δ-meso methyl incorporated at day 4 (for example), then the peak heights observed in Figure 2 would not be proportional, and the quantitative results from a ^{13}C-pulsed feeding experiment would be fundamentally uninterpretable.

We also separated the [^{14}C]glutamate- and [^{14}C]glycine-derived Bmphs into their individual homologues and determined the relative specific activity of each homologue. The specific activity was obtained by collecting the HPLC column effluent, counting the radioactivity in each peak, and dividing by the integrated UV_{405} peak area. HPLC chromatograms of the glycine- and glutamate-derived 5-Et-Bmphs are given in Figures 3 and 4, respectively, with both the UV_{405} and the radioactive tracings shown. One would predict that the specific activities of the glutamate-labeled Bmphs, where incorporation is seen only in the chlorin macrocycle, would remain constant over the entire range of alkyl homologues. In the glycine-labeled Bmphs, on the other hand, one would expect the specific activities of the homologues to increase linearly with respect to the total number of methionine-derived methyls in each homologue. And this is the result that was generally observed, except for some slight but biosynthetically significant variations. For the glutamate-labeled Bmphs, the observed specific activities for the 5-Et and 5-Me series are plotted in Figure 5, and it can be clearly seen that the values are fairly constant at 0.94 ± 0.13 for the 5-Et and 1.44 ± 0.13 for the 5-Me series. However, an obvious question that arises from these data is, why is there such a large discrepancy between the average specific activities observed in the 5-Et and 5-Me compounds? One possible explanation is a systematic experimental error, since the 5-Et and 5-Me series were separated by TLC prior to HPLC separation, and so the 5-Et and 5-Me specific activity data sets were obtained from separate HPLC

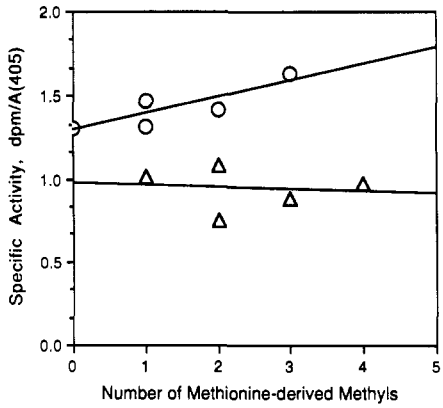

FIGURE 5: Plot of specific activity of Bmphs from [1-^{14}C]glutamate feeding vs number of methionine-derived methyls for each separated homologue. (○) 5-Me series; (△) 5-Et series.

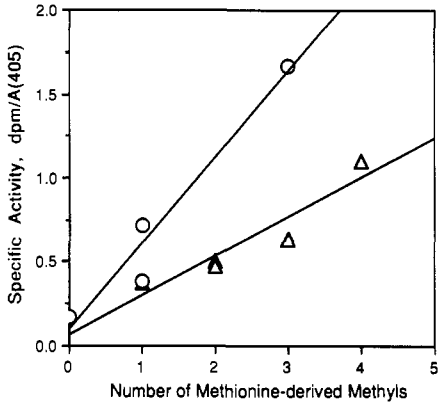

FIGURE 6: Plot of specific activity of Bmphs from [2-^{14}C]glycine feeding vs number of methionine-derived methyls for each separated homologue. (○) 5-Me series; (△) 5-Et series.

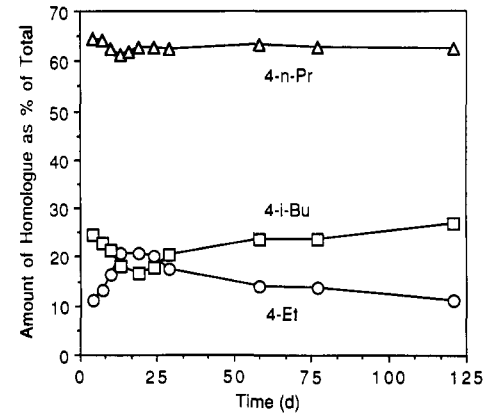

FIGURE 7: Plot of percentage homologue composition vs elapsed time of culture growth for *Chlb* strain D. Homologues were separated by HPLC, and then amounts were estimated spectrophotometrically.

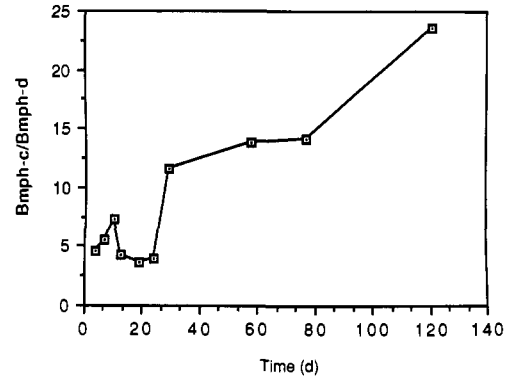

FIGURE 8: Plot of ratio of Bmph-c/Bmph-d vs elapsed time of culture growth for *Chlb* strain D. Homologues were separated by HPLC, and then amounts were estimated spectrophotometrically.

runs. Alternatively, the discrepancy could be real, resulting from an actual shift by the organism toward synthesis of the less alkylated 5-Me-BChls during the period in the growth curve from $t = 0$ to 4 days.

On examining a similar plot of specific activity vs number of methionine-derived methyls in each homologue for the [2-^{14}C]glycine experiment (Figure 6), we can also see that, as expected, the values increase somewhat linearly over the range of the homologues. And although the 5-Me data are not as linear as the model would have predicted, it is obvious that the slope of this curve is much greater than the slope of the 5-Et curve, adding further evidence of preferential production of the 5-Me-BChls during the first 4 days of the growth curve. This hypothesis could also account for the observed positive y intercept in both curves, indicating a similar shift toward less R^4 and δ-meso alkylation during the initial period of growth.

Time-Course Studies of Bmph Alkylation. Intrigued by earlier observations that the homologue composition of the *Chlb* 8327 Chls had changed over a period of several years (Smith & Bobe, 1987; Bobe et al., 1990), we decided to probe the changes in homologue production using HPLC and pulsed feedings with [2-^{13}C]glycine. HPLC analysis of Bmphs extracted from aliquots of bacteria taken from a 20-L carboy of *Chlb* strain D over a 120-day period provided additional evidence in support of the light adaptation hypothesis (Bobe et al., 1990; Smith & Bobe, 1987). A plot of percent homologue composition vs time (Figure 7) shows a decline in R^4 alkylation from the time of subculturing until about 20 days, presumably a response by the organism to the greater availability of light upon subculturing from a mature steady state to a diluted situation. As the bacterial culture grows and becomes more opaque, the bacteria shift slowly toward production of more alkylated homologues, finally returning to the composition of the original mature mother culture. Similarly, a plot of the Bmph-c/Bmph-d ratio (Figure 8) shows a decline in meso alkylation, followed by a steady increase in Bmph-c to about 95% of the total Bmphs. The fact that BChl methylation can be increased or decreased by the organism in response to environmental factors indicates that this is not a simple mutation (in which case one would expect to see complete and irreversible methylation), but rather an adaptable mechanism which is used by the bacterium as needed. In this case, however, it is not possible to differentiate between an adaptation of biosynthesis to fit external conditions and a simple selection between the BChl-c and BChl-d producing bacteria in the medium.

This phenomenon can also be seen in ^{13}C NMR results from pulsed feedings of *Chlb* strain D (Figure 9), where several 1-L cultures were inoculated at the same time and then fed with [2-^{13}C]glycine at ages of 0, 7, 14, and 21 days (Figure 9A–D), followed by harvest and Bmph extraction 5 days later. The fifth culture (Figure 9E) was fed with [^{13}C-CH_3]-methionine at $t = 38$ days and harvested 75 days later, but it still serves to illustrate the general trends observed. In the first and second spectra (Figure 9A,B), the large 5b-Et/meso-Me peak height ratios indicate a rapid shift toward BChl-d production during the lag and exponential phases of growth. Similarly, a sharp decrease in the 4c-*n*-Pr signal (Figure 9B) reflects greater production of the less alkylated 4-Et homologues. After 2–3 weeks (Figure 9C,D) the degree of meso methylation has increased considerably, while 5b-alkylation dips briefly and 4-*i*-Bu production nearly vanishes, possibly as a metabolic compensation mechanism to allow more meso methylation.

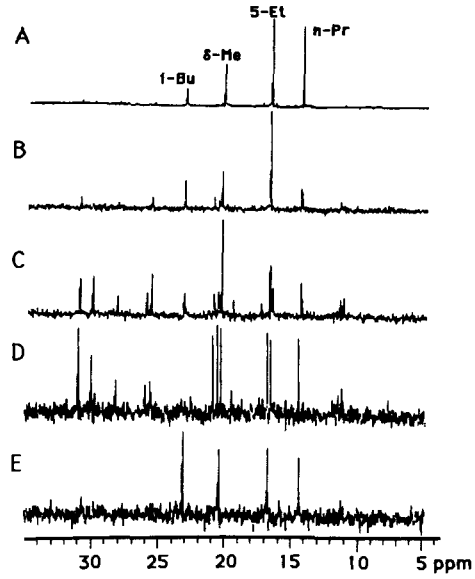

FIGURE 9: 75-MHz ^{13}C NMR spectra from ^{13}C-pulsed feeding experiment. One-liter cultures of *Chlb* strain D were fed at age (A) 0 days, (B) 7 days, (C) 14 days, (D) 21 days, and (E) 38 days.

This would be during the logarithmic and early stationary phases of growth, where more of the new BChl is destined for replacement (i.e., turnover) of the older BChl. Finally, the long-term methionine feeding (Figure 9E) indicates stabilization of the 5b-Et/meso-Me ratio (i.e., nearly complete meso and 5 methylation), while R^4 alkylation has increased as expected, as seen by greater enrichment of 4c-*i*-Bu and 4c-*n*-Pr peaks.

Another trend observed is the gradual decline in net enrichment of the methionine-derived methyl signals over the first 3 weeks, reflecting the expected decrease in BChl biosynthesis as the culture reaches stationary phase. This is consistent with reports (Hendry & Stobart, 1986) that chlorophyll turnover rates are inversely proportional to age in several green plant species. In another experiment to estimate extent of BChl turnover, cultures were fed with [^{14}C-CH_3]-methionine at $t = 0$ days, and aliquots were removed at various times during the stationary phase. BChl turnover was indicated by a decrease in specific activity (not shown), as older radiolabeled BChl molecules were replaced by new unlabeled or less labeled ones. It was also apparent that this rate of turnover declined with age, in all strains of *Chlb* bacteria examined.

In retrospect, it should be noted that methionine is certainly a better probe of BChl methylation than is glycine. Methionine is a more directed metabolic precursor, whereas glycine is extensively metabolized and diluted into the carbon pool. It was for this reason that methionine was used in the long-term pulsed feeding experiment (Figure 9E), giving enrichment entirely in the four extra methyl carbons.

Conclusions

Feeding studies with glutamate, glycine, and methionine have demonstrated operation of the C_5 and C_1 metabolic pathways in *Chlb* strain D BChl biosynthesis, and confirmed the C_1 pathway in *P. aestuarii*. In addition, the evidence confirms that the bacteria can adjust the homologue composition of its antenna BChls in response to varying growth conditions. The timing of these changes within a single culture (Figures 7 and 8) is certainly consistent with a light adaptation mechanism, which would predict that degree of alkylation is directly proportional to cell density (i.e., self-shading of the cells) in the culture flask. While light adaptation may be a valid explanation for the gradual changes seen in BChl alkylation over a long period of time with intermittent subculturing, there may be other factors influencing pigment composition during the lifespan of a single culture. One important factor may be the diversion of glycine, methionine, and other C_1 precursors into synthesis of proteins and other cell constituents during the initial period of growth, when cell mass is increasing most rapidly. This might account for the observed dip in BChl methylation during the initial period of growth. During the stationary phase, when cell growth has slowed or stopped, more methionine is available for BChl methylation, so that the older, less alkylated BChl is turned over and replaced by the more highly alkylated homologues.

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Electron Transfer between Primary and Secondary Donors in *Rhodospirillum rubrum*: Evidence for a Dimeric Association of Reaction Centers†

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ABSTRACT: Light-induced oxidation of the primary electron donor P and of the secondary donor cytochrome c_2 was studied in whole cells of *Rhodospirillum rubrum* in the presence of myxothiazole to slow down their reduction. 1. The primary and secondary electron donors are close to thermodynamic equilibrium during continuous illumination when the rate of the electron transfer is light-limited. This implies a long-range thermodynamic equilibration involving the diffusible cytochrome c_2. A different behavior is observed with *Rhodobacter sphaeroides* R26 whole cells, in which the cytochrome c_2 remains trapped within a supercomplex including reaction centers and the cytochrome b/c complex [Joliot, P., et al. (1989) *Biochim. Biophys. Acta 975*, 336-345]. 2. Under weak flash excitation, the reduction kinetics of the photooxidized primary donor are nearly exponential with a half-time in the hundred microseconds time range. 3. Under strong flash excitation, the reduction of the photooxidized primary donor follows a second-order kinetics. About half of the photooxidized primary donor is reduced in a few milliseconds while the remainder stays oxidized for hundreds of milliseconds despite an excess of secondary donors in their reduced form. The flash intensity dependence of the amplitude of the slow phase of P^+ reduction is proportional to the square of the fraction of reaction centers that have undergone a charge separation. These results are correctly described with a model in which (1) reaction centers are associated in dimers, (2) the affinity of cytochrome c_2 for the reaction center is low when the cytochrome c_2 is reduced but high when oxidized, and (3) electrostatic or steric interactions prevent the binding of two cytochrome c_2 molecules on the same dimer P-P.

Stabilization of the oxidoreduction products after the light-induced charge separation involves subsequent reactions with secondary acceptors and donors. In purple bacteria, two types of *c* cytochromes operate as direct electron donors to the photooxidized primary donor P (Dutton & Prince, 1978). For species such as *Rhodopseudomonas viridis* or *Chromatium vinosum*, the *c* cytochrome contains four hemes and is tightly bound to the reaction center complex, leading to very fast electron donation at room temperature ($t_{1/2} \approx 300$ ns in the case of *Rp. viridis*; Dracheva et al., 1986; Shopes et al., 1987). This reaction remains efficient even at helium temperature (Dutton, 1971; Rutherford et al., 1979; Verméglio et al., 1989; Hubbard & Evans, 1989). In the case of *Rhodobacter* (*Rb.*)[1] *sphaeroides* and *Rhodospirillum* (*Rs.*) *rubrum* species, the photooxidized primary electron donor is reduced by a soluble cytochrome (cyt) c_2 (Dutton & Prince, 1978). This reaction is blocked at temperatures lower than 240 K (Vredenberg & Duysens, 1964). Two phases ($t_{1/2} \approx 3$ μs and 100 μs-1 ms) are observed at room temperature for whole cells (Overfield et al., 1979), chromatophores (Dutton et al., 1975), or purified reaction centers and cyt c_2 of *Rb. sphaeroides* (Dutton & Prince, 1978; Overfield et al., 1979; Overfield & Wraight, 1980). Several lines of evidence indicate that the fast phase is due to cyt c_2 bound to the reaction center (Dutton & Prince, 1978; Overfield et al., 1980), while the effect of the viscosity and ionic strength on the slow phase implies that this process is diffusional (Dutton & Prince, 1978; Overfield et al., 1979; Prince et al., 1974; Van der Wal et al., 1986). We recently showed in *Rb. sphaeroides* R26 whole cells that the diffusion of one molecule of cyt c_2 is restricted to a domain including two reaction centers and one cyt bc_1 complex forming a "supercomplex" (Joliot et al., 1989).

Oxidation of cyt c_2 in *Rs. rubrum* cells is slower by more than 1 order of magnitude than in *Rb. sphaeroides* (Van Grondelle et al., 1976). Measuring the kinetics of cyt c_2 oxidation as a function of the flash intensity or of the viscosity

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[1] Abbreviations: cyt, cytochrome; cyt b/c_1 complex, ubiquinol-cytochrome $c(c_2)$ oxidoreductase; *Rb.*, *Rhodobacter*; *Rs.*, *Rhodospirillum*.

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