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Anaerobic Protoporphyrin Biosynthesis does not Require Incorporation of Methyl Groups from Methionine

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Anaerobic Protoporphyrin Biosynthesis Does Not Require Incorporation of Methyl Groups from Methionine

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It was recently reported (H. Akutsu, J.-S. Park, and S. Sano, J. Am. Chem. Soc. 115:12185–12186, 1993) that in the strict anaerobe *Desulfovibrio vulgaris* methyl groups from exogenous L-methionine are incorporated specifically into the 1 and 3 positions (Fischer numbering system) on the heme groups of cytochrome c_3. It was suggested that under anaerobic conditions, protoporphyrin IX biosynthesis proceeds via a novel pathway that does not involve coproporphyrinogen III as a precursor but instead may use precorrin-2 (1,3-dimethyluroporphyrinogen III), a siroheme and vitamin B_{12} precursor which is known to be derived from uroporphyrinogen III via methyl transfer from *S*-adenosyl-L-methionine. We have critically tested this hypothesis by examining the production of protoporphyrin IX-based tetrapyrroles in the presence of exogenous [^{14}C]methyl-L-methionine under anaerobic conditions in a strict anaerobe (*Chlorobium vibrioforme*) and a facultative anaerobe (*Rhodobacter capsulatus*). In both organisms, ^{14}C was incorporated into the bacteriochlorophyll precursor, Mg-protoporphyrin IX monomethyl ester. However, most of the label was lost upon base hydrolysis of this compound to yield Mg-protoporphyrin IX. These results indicate that although the administered [^{14}C]methyl-L-methionine was taken up, converted into *S*-adenosyl-L-methionine, and used for methyl transfer reactions, including methylation of the 6-propionate of Mg-protoporphyrin IX, methyl groups were not transferred to the porphyrin nucleus of Mg-protoporphyrin IX. In other experiments, a *cysG* strain of *Salmonella typhimurium*, which cannot synthesize precorrin-2 because the gene encoding the enzyme that catalyzes methylation of uroporphyrinogen III at positions 1 and 3 is disrupted, was capable of heme-dependent anaerobic nitrate respiration and growth on the nonfermentable substrate glycerol, indicating that anaerobic biosynthesis of protoporphyrin IX-based hemes does not require the ability to methylate uroporphyrinogen III. Together, these results indicate that incorporation of L-methionine-derived methyl groups into porphyrins or their precursors is not generally necessary for the anaerobic biosynthesis of protoporphyrin IX-based tetrapyrroles.

Biosynthesis of porphyrins and related compounds proceeds via a common set of intermediates through the first cyclic tetrapyrrole, uroporphyrinogen III, at which point the pathway splits into one branch leading to siroheme, vitamin B_{12}, and other reduced tetrapyrrole end products and another branch leading to oxidized end products, including hemes, bilins, chlorophylls, and bacteriochlorophylls (Fig. 1).

The reduced tetrapyrrole end products are distinguished by their possession of methionine-derived methyl groups. Recent genetic and biochemical analysis of the biosynthesis of reduced tetrapyrroles in *Escherichia coli* and *Salmonella typhimurium* has revealed that a single protein, the product of the *cysG* gene, catalyzes all of the steps of siroheme formation from uroporphyrinogen III (8, 20, 23). These steps consist of (i) the transfer of methyl groups from *S*-adenosyl-L-methionine to positions 1 and 3 of the tetrapyrrole to form precorrin-2, (ii) the transfer of two electrons from the tetrapyrrole to NAD^+, and (iii) the insertion of Fe^{2+} into the macrocycle.

It is generally believed that the first biosynthetic step of the branch leading to the oxidized end products is decarboxylation of the four acetate groups of uroporphyrinogen III to methyl groups, yielding coproporphyrinogen III. This reaction is catalyzed by uroporphyrinogen III decarboxylase (EC 4.1.1.37), the product of the *hemE* gene (13). The next step is oxidative decarboxylation of the propionate groups at positions 2 and 4 to vinyl groups, yielding protoporphyrinogen IX.

In obligately aerobic organisms, coproporphyrinogen III oxidation is an O_2-requiring reaction catalyzed by coproporphyrinogen III oxidase (EC 1.3.3.3). There have been very few reports of anaerobic coproporphyrinogen III oxidation. Ehteshamuddin (6) reported that coproporphyrinogen III was converted to protoporphyrin IX both aerobically and anaerobically in extracts of aerobically grown *Pseudomonas* sp. cells, in reaction mixtures supplemented with Mg^{2+} and reduced glutathione. Tait (21) showed that anaerobic coproporphyrinogen III oxidation in extracts of *Rhodobacter sphaeroides* and *Chromatium* strain D required Mg^{2+}, ATP, NAD^+, and L-methionine. Similar requirements were reported for anaerobic conversion of coproporphyrinogen III to protoporphyrinogen by extracts of *Saccharomyces cerevisiae* mitochondria (15). The mechanistic role of L-methionine in the anaerobic reaction was not explained. More recently, Seehra et al. (17) have studied the anaerobic as well as the aerobic formation of protoporphyrinogen IX from porphobilinogen in extracts of *R. sphaeroides*. For the reaction to proceed anaerobically beyond coproporphyrinogen III, it was necessary to supplement the incubation mixture with L-methionine. The authors drew the conclusion that L-methionine is required for the anaerobic coproporphyrinogen III oxidase reaction. However, the experiments relied on in situ coupled reactions beginning with porphobilinogen, and the direct conversion of coproporphyrinogen III to protoporphyrinogen IX was not determined. Therefore, the possibility that porphobilinogen or uroporphyrinogen III is con-

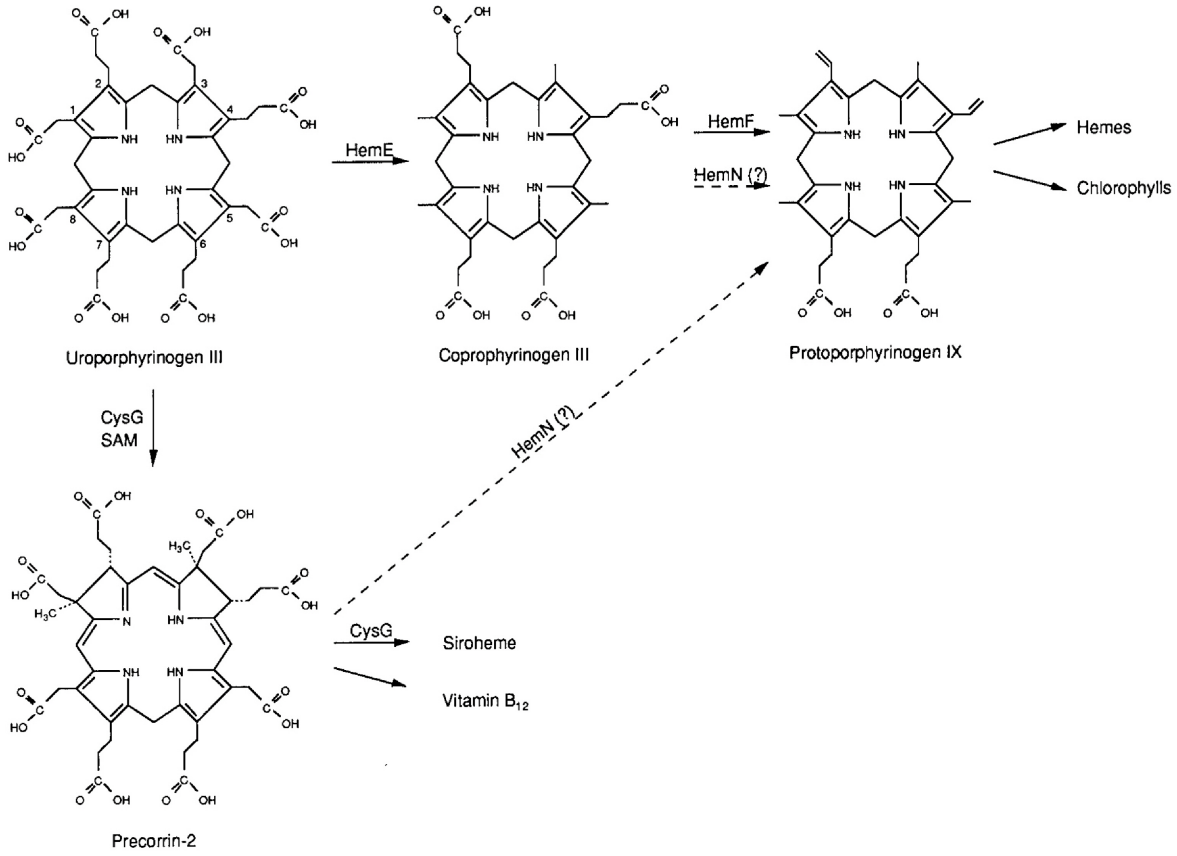

FIG. 1. Biosynthetic pathways from uroporphyrinogen III to protoporphyrinogen IX and precorrin-2. The Fischer numbering system for tetrapyrrole substituent positions is shown for uroporphyrinogen III.

verted to protoporphyrinogen IX by some other, uncharacterized, methionine-dependent mechanism could not be excluded.

Genetic analysis of *S. typhimurium* and *E. coli* has revealed the existence of two genes, *hemF* and *hemN*, involved in protoporphyrinogen IX formation. Either gene product alone is sufficient for heme synthesis under aerobic conditions, since single *hemF* and *hemN* mutants each retain a Hem^+ phenotype aerobically, whereas only *hemF hemN* double mutants are Hem^- under these conditions (22, 25, 27). The *hemF* gene encodes a coproporphyrinogen III oxidase similar to the yeast O_2-dependent enzyme (26). The HemF enzyme has not been purified, but its activity in unfractionated cell extracts requires O_2. In contrast, the *hemN* gene is required for oxygen-independent protoporphyrinogen IX formation. This requirement is revealed genetically by the Hem^- phenotype of *hemN* mutants during anaerobic growth, during which the HemF enzyme cannot function, or by that of a *hemF hemN* double mutant during aerobic growth. Although it has been assumed that HemN catalyzes anaerobic oxidation of coproporphyrinogen III, activity of HemN has not been detected in vitro.

A possible new explanation for the L-methionine requirement in anaerobic protoporphyrinogen IX formation, as well as for the difficulty in demonstrating anaerobic coproporphyrinogen III oxidase activity in cell extracts, was recently provided by an unexpected finding by Akutsu et al. (1). These workers reported that in the strict anaerobe *Desulfovibrio vulgaris*, methyl groups from exogenous L-methionine are incorporated specifically into the 1 and 3 positions on the heme groups of cytochrome c_3. It was suggested that under anaerobic conditions, protoporphyrin IX biosynthesis proceeds via a novel pathway that does not involve coproporphyrinogen III but instead may use precorrin-2 as a precursor. We have attempted to find evidence to support this hypothesis by examining the incorporation of label from exogenous [^{14}C]methylmethionine into protoporphyrin IX-derived tetrapyrroles in anaerobically growing cells of the strict anaerobe *Chlorobium vibrioforme* and the facultative anaerobe *Rhodobacter capsulatus*. We have also sought genetic support for the hypothesis by determining whether *S. typhimurium cysG* mutant cells, which cannot methylate uroporphyrinogen III, are capable of forming hemes anaerobically.

MATERIALS AND METHODS

S. typhimurium. All procedures for growth, mutagenesis, construction, and phenotype determination of *S. typhimurium* strains were done as previously described (3, 8, 25–27).

Photosynthetic bacterial strains and cell culture. *C. vibrioforme* f. sp. *thiosulfatophilum* 8327 was grown in the medium of Sirevåg and Ormerod (18) as described by Rieble et al. (16) in completely filled 60-ml bottles with rubber serum bottle stoppers as caps. When the cells were in late logarithmic growth phase (A_{640} = 0.6), the culture was supplemented with 30 μl of Ethrel (a commercial solution of 2-chloroethylphosphonic acid), 30 μl of 5 N NaOH, and 10 μCi of [^{14}C]methyl-L-methionine, which were added by injection through the serum bottle stopper. After 3 h, the culture was cooled on ice before the cells were harvested as described below.

The *R. capsulatus* mutant strain DB575 (*orf575*::Km^r *rif-10*), which accumulates Mg-protoporphyrin IX monomethyl ester (MPE) (4), was grown overnight to stationary phase in RCV 2/3 PY medium (29) at 34°C. The cells were then subcultured 1:170 in CA medium (11) supplemented with 20 mM fructose, 40 mM dimethyl sulfoxide (10), and 10 μCi of [^{14}C]methyl-L-methionine and grown

anaerobically for 24 h in the dark at 34°C. The culture was then cooled on ice before the cells were harvested as described below.

Pigment extraction. *C. vibrioforme* pigments were extracted as described by Ormerod et al. (14). A 60-ml culture was harvested by centrifugation (8,000 × *g*, 5 min), and the cells were resuspended in 6 ml of cold acetone and then resedimented. The acetone supernatant was decanted, 3 ml of hexanes and 1.5 ml of 0.1 N NH_4OH were added with mixing, and the phases were allowed to separate. The acetone phase was reserved and extracted first with 2 ml of hexanes and then with 2 ml of 2-methylbutane. Residual 2-methylbutane was removed from the acetone phase by exposure to an N_2 stream. Saturated aqueous NaCl (1.7 ml) was added, and the pH was adjusted to 6.5 with 0.25 M maleic acid (monosodium salt). The pigments were extracted with two 3-ml portions of diethyl ether, which were combined and then divided into 3-ml fractions, each of which was concentrated to a 300-μl final volume.

R. capsulatus cells from 34 ml of medium were harvested by centrifugation (8,000 × *g*, 5 min). The cell pellet was resuspended in 1 ml of 0.1 N NH_4OH, and 4 ml acetone was added. This suspension was heated for 5 min at 65°C and then cooled on ice and recentrifuged. The acetone supernatant was decanted and extracted twice with 0.5 volumes of hexanes and 0.5 volumes of 2-methylbutane. To the remaining acetone phase, approximately 0.06 volumes of saturated aqueous NaCl was added, and the solution was adjusted to pH 6.5 by the addition of 0.25 M maleic acid (monosodium salt). The pigments were then extracted into diethyl ether by two 1-ml extractions. The sample was concentrated to 400 μl, and 20-μl aliquots were used for high-performance liquid chromatography (HPLC) as described below.

Base hydrolysis of MPE. Base hydrolysis of MPE to yield Mg-protoporphyrin IX (MP) was done as described by Chereskin and Castelfranco (5). The collected MPE-containing HPLC eluate in 1 ml of 70% (vol/vol) aqueous methanol was adjusted to 5.0 ml with the addition of 3.25 ml of methanol and 750 μl of 10 N KOH, to reach final concentrations of 80% methanol and 1.5 N KOH. The mixture was incubated overnight in the dark at room temperature, and then 2.5 ml of H_2O and 3.75 ml of saturated aqueous NaCl were added and the pH was adjusted to 6.5 by adding 15 ml of 0.5 M maleic acid (monosodium salt). Pigments were extracted with two 10-ml portions of diethyl ether, the combined diethyl ether extract was concentrated to 1 ml, and water was removed by freezing overnight at −20°C, followed by decantation from the ice. The solution was concentrated to a final volume of 10 μl by evaporation under an N_2 stream and used for HPLC.

HPLC. HPLC was performed as previously described (7). Samples in diethyl ether were diluted to 100 μl with methanol–5 mM aqueous tetrabutylammonium phosphate (7:3, vol/vol), and aliquots were injected onto a Zorbax ODS column (4.6 by 100 mm; Du Pont, Wilmington, Del.) preequilibrated with methanol–5 mM aqueous tetrabutylammonium phosphate (7:3, vol/vol). Elution with this solvent was continued for 3 min at a flow rate of 1.0 ml/min, and then the solvent was changed to methanol-water (7:3, vol/vol). Elution was monitored with a Fluorichrom fluorescence detector (Varian, Sunnyvale, Calif.). Eluate fractions were collected at 1-min intervals for analysis of radioactivity and fluorescence spectra. Because slight variations in the elution solvent composition influence the elution times of the pigments, the column was calibrated with samples of authentic MP and MPE for each set of analyses by using the same preparation of elution solvent as was used for the experimental samples.

Thin-layer chromatography. Thin-layer chromatography of the diethyl ether extracts was done on silica gel plates. The elution solvent was toluene-ethyl acetate-ethanol (4:1:1, vol/vol/vol). Migration of tetrapyrroles was monitored by fluorescence from long-wavelength UV illumination. For determination of radioactivity, the fluorescent spots were scraped directly into scintillation vials.

Fluorescence spectroscopy. Fully corrected fluorescence emission spectra of fractions collected from the HPLC were determined in 70% (vol/vol) aqueous methanol with a fluorescence spectrophotometer (Spex Industries, Metuchen, N.J.) by using an excitation wavelength of 418 nm. Quantitation of MP and MPE was done by use of dilutions of a standard MP solution, the concentration of which was calculated from absorption at 419 nm in diethyl ether by using an absorption coefficient of 308,000 M^{-1} (9).

Chemicals. MPE was a generous gift from P. A. Castelfranco (University of California, Davis). Ethrel was a gift from Union Carbide Agricultural Products (Research Triangle Park, N.C.). MP was purchased from Porphyrin Products (Logan, Utah). [^{14}C methyl-L-methionine (specific radioactivity, 40 Ci/mol) was from ICN Biochemicals (Irvine, Calif.). Tetrabutylammonium phosphate was from Rainin Instruments (Woburn, Mass.). Silica gel G-HL plates (Universal Absorbents) were from Fisher Scientific (Pittsburgh, Pa.). All other chemicals were from Fisher, Sigma Chemical Co. (St. Louis, Mo.), or Research Organics (Cleveland, Ohio).

RESULTS AND DISCUSSION

C. vibrioforme. *C. vibrioforme*, a strictly anaerobic green phototrophic bacterium, was a useful organism for these experiments because it was previously shown that *C. vibrioforme* cells rapidly accumulate large amounts of the bacteriochlorophyll precursor, MPE, when they are exposed to any of several anesthetic gases such as ethylene, acetylene, and N_2O (14). MPE accumulation was induced in the presence of exogenous [^{14}C]methyl-L-methionine by administration of Ethrel, which hydrolyzes in situ to produce ethylene. The [^{14}C]methyl-L-methionine that was administered to a 60-ml culture had a total radioactivity of 10 μCi and a specific radioactivity of 40 Ci/mol. After 3 h, the cells were harvested and extracted, and a sample equal to 6.7% of the total pigment extract was analyzed by HPLC. The major fluorescent pigment eluted at 17 to 18 min, a time corresponding to that of authentic MPE (Fig. 2). The major portion of the radioactivity was also associated with this elution time. This region of the HPLC eluate (fractions 17 and 18) had a fluorescence emission spectrum that was similar (emission peaks at 596 and 663 nm), but not identical, to that of authentic MPE (emission peaks at 596 and 650 nm) (Fig. 3). The difference between the two spectra in the 650- to 665-nm region was caused by the presence of a contaminating pigment in the MPE-containing region of the eluate from the cell extract (see below). This pigment fluoresces at 666 nm but not at 596 nm. From the emission intensity at 596 nm, it was calculated that the HPLC eluate region contained 1.31 nmol of MPE.

A second sample, equal to 10% of the total pigment extract, was injected onto the HPLC column. Eluate fractions 17 and 18 were collected, analyzed by fluorescence spectrophotometry, and then base hydrolyzed overnight and reanalyzed by HPLC. Two fluorescent peaks were detected. The first eluted at 9 min and had both an elution time and a fluorescence emission spectrum corresponding to those of authentic MP. The second peak had an elution time corresponding to that of

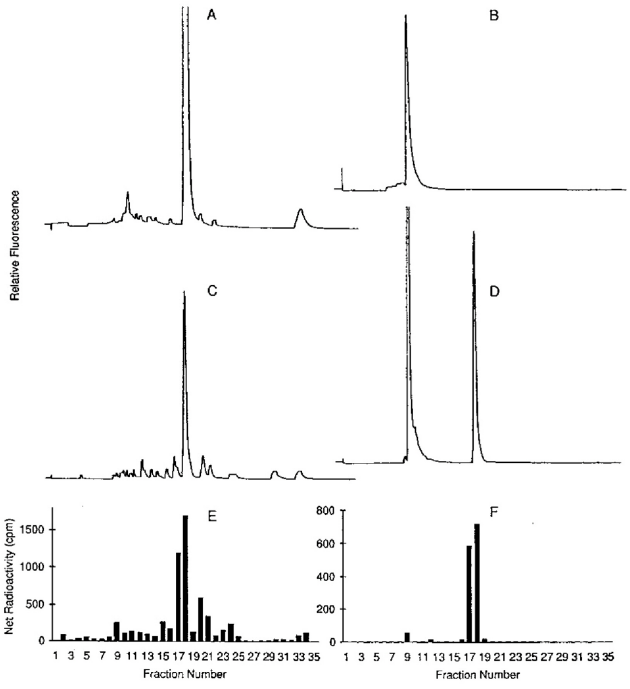

FIG. 2. HPLC elution of *C. vibrioforme* pigment extracts. (A) Fluorescence emission profile of the eluate after injection of standard MPE; (B) fluorescence emission profile of the eluate after injection of standard MP; (C) fluorescence emission profile of the eluate after injection of *C. vibrioforme* extract; (D) fluorescence emission profile of the eluate after injection of base-hydrolyzed material collected from fractions 17 and 18 of the HPLC profile shown in panel C; (E) radioactivity in eluate fractions collected from the injection shown in panel C; (F) radioactivity in eluate fractions collected from the injection shown in panel D.

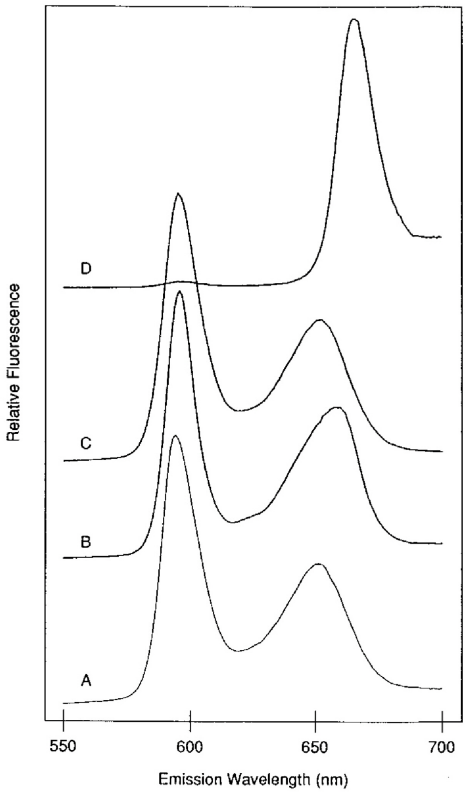

FIG. 3. Fully corrected fluorescence emission spectra of standard MPE (A), material collected in fractions 17 and 18 of the HPLC profile shown in Fig. 2C (B), material collected in fraction 9 of the HPLC profile shown in Fig. 2D (C), and material collected in fractions 17 and 18 of the HPLC profile shown in Fig. 2D (D).

MPE. However, the fluorescence emission spectrum did not resemble that of MPE but instead had a single peak at 666 nm. The identity of this peak was not investigated further. However, on the basis of its incorporation of non-base-hydrolyzable ^{14}C from [^{14}C]methyl-L-methionine and its fluorescence emission spectrum, it is reasonable to conclude that it consisted of, or was derived from, chlorophyllides obtained from base hydrolysis of chlorosome chlorophylls (bacteriochlorophylls *c* and *d*) which are the major pigments of *C. vibrioforme*. These pigments absorb light maximally at 650 to 660 nm and contain as many as five L-methionine-derived nonester methyl groups (19).

From the fluorescence emission intensity at 596 nm and the radioactivity in the collected sample eluting at 9 min, it was determined that this sample contained 0.91 nmol of MP and 54 net cpm of radioactivity and that the specific radioactivity was 59 cpm/nmol. To calculate the specific radioactivity of the MPE in the sample before base hydrolysis, it was necessary to determine the portion of the radioactivity in the HPLC eluate fraction that was incorporated into MPE. This was accomplished by chromatography of a sample of the HPLC eluate fraction on a silica gel thin-layer plate. This procedure yielded two pigment fractions, one of which coeluted with authentic MPE (data not shown). The pigment spots were transferred to scintillation vials, and their radioactivity was determined. Of a total of 2,358 cpm of radioactivity in the applied sample, 732 cpm was contained in the MPE spot. The specific radioactivity of the MPE was therefore 732 cpm/1.31 nmol, or 559 cpm/nmol.

If the 559-cpm/nmol specific radioactivity on MPE were distributed equally in three methyl groups of MPE, those at positions 1 and 3 of the tetrapyrrole and the methyl ester of the 6-propionate, then the MP that was derived from this MPE by base hydrolysis would be expected to have a specific radioactivity of 373 cpm/nmol, which is two-thirds of the value for MPE. The actual specific radioactivity of the MP, 59 cpm/nmol, was only 11% of the value for MPE and less than 16% of the expected value.

It is clear from the analysis presented above that the administered [^{14}C]methyl-L-methionine was taken up, converted into *S*-adenosyl-L-methionine, and used for methyl transfer reactions, including methylation of the 6-propionate of MP as well as the nonester methyl groups of bacteriochlorophylls. However, methionine methyl groups were not transferred to the porphyrin nucleus of MP to a significant extent. The level of radioactivity in the MP was far below the value to be expected for direct transfer of methyl groups from methionine, and it was probably attributable to indirect, secondary incorporation of label derived from catabolism of methionine.

R. capsulatus. An experiment similar to the one described above for *C. vibrioforme* was done. For *R. capsulatus*, instead of inducing MPE accumulation by administration of Ethrel, we used a mutant strain that accumulates MPE because of a defect in an enzyme of the bacteriochlorophyll biosynthetic pathway. The [^{14}C]methyl-L-methionine that was administered to 34 ml of culture had a total radioactivity of 10 μCi and a specific radioactivity of 40 Ci/mol. After 24 h of anaerobic growth in the dark, the cells were harvested and extracted, and a sample equal to 5% of the total pigment extract was analyzed by HPLC. There were four major fluorescent, radioactive peaks (Fig. 4). The peak that eluted at 15 min had an elution time and a fluorescence emission spectrum identical to those of authentic MPE (data not shown). This peak was collected for further analysis, and the other peaks were not analyzed further.

A second sample, equal to 5% of the total pigment extract, was injected onto the HPLC column. Eluate fraction 16 was collected, analyzed by fluorescence spectrophotometry, and then base hydrolyzed overnight and reanalyzed by HPLC. A single fluorescent peak with an elution time of 7 min was detected. The peak had an elution time and a fluorescence emission spectrum identical to those of authentic MP (data not shown).

From the fluorescence emission intensity at 596 nm and the radioactivity in the collected prehydrolysis sample eluting at 15 min, it was determined that this sample contained 33 pmol of MPE and 1,138 net cpm of radioactivity and that the specific radioactivity was 34,485 cpm/nmol. Similarly, from the fluorescence emission intensity at 596 nm and the radioactivity in the collected posthydrolysis sample eluting at 9 min, it was determined that this sample contained 16.5 pmol of MP and 74 cpm of radioactivity and that the specific radioactivity was 4,485 cpm/nmol.

If the 34,485-cpm/nmol specific radioactivity on MPE were distributed equally in three methyl groups of MPE, those at positions 1 and 3 of the tetrapyrrole and the methyl ester of the 6-propionate, then the MP that was derived from this MPE by base hydrolysis would be expected to have a specific radioactivity of 22,990 cpm/nmol, which is two-thirds of the value of MPE. The actual specific radioactivity of the MP, 4,485 cpm/nmol, was only 13% of the value for MPE and less than 20% of the expected value.

The analysis presented above shows that, as was the case for *C. vibrioforme*, the administered [^{14}C]methyl-L-methionine was taken up, converted into *S*-adenosyl-L-methionine, and used

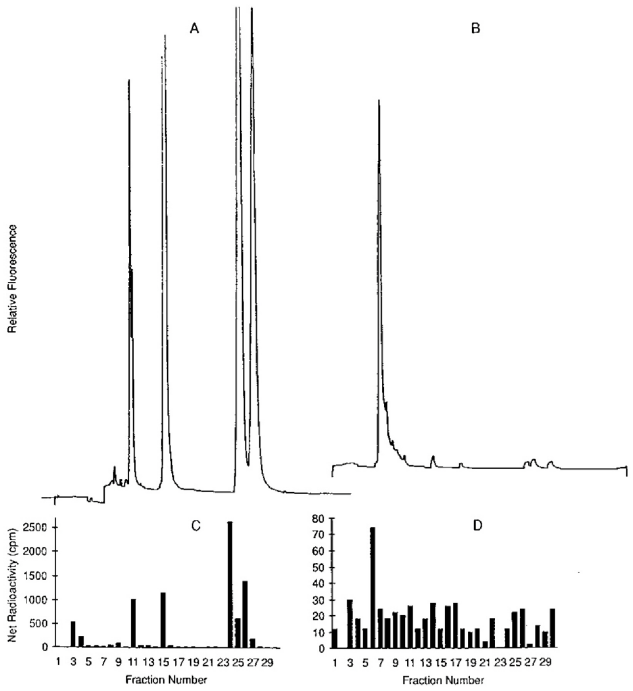

FIG. 4. HPLC elution of *R. capsulatus* pigment extracts. (A) Fluorescence emission profile of the eluate after injection of *R. capsulatus* extract; (B) fluorescence emission profile of the eluate after injection of base-hydrolyzed material collected from fraction 15 of the HPLC profile shown in panel A; (C) radioactivity in eluate fractions collected from the injection shown in panel A; (D) radioactivity in eluate fractions collected from the injection shown in panel B.

for methyl transfer reactions, including methylation of the 6-propionate of MP. However, as for *C. vibrioforme*, ^{14}C was not present in the porphyrin nucleus of MP to the extent required to support the hypothesis that porphyrin-methyl groups are derived from methionine-methyl.

S. typhimurium. The hypothesis to be tested was that in addition to its known roles in siroheme and cobalamin synthesis (8, 20, 23, 24), the *cysG*-encoded uroporphyrinogen III 1,3-methylase is also required for the anaerobic *hemN* route of protoporphyrinogen IX formation and accounts for the presence of methionine-derived methyl groups at positions 1 and 3 of cytochrome c_3 hemes in *D. vulgaris* (1). It would be expected that if the CysG enzyme plays such a role, the associated phenotypes would have been noted in previous studies of *cysG* mutants. However, Barrett and Chang (2) tested the requirement of *cysG* mutants for L-cysteine during anaerobic growth on minimal glucose agar, for which heme is not required. Goldman and Roth (8) also grew *cysG* mutants anaerobically on minimal glucose medium. In a previous study, only *hemN* mutants were recovered in a search for strains defective in the alternate pathway (25), but this search was carried out on minimal glycerol agar. Any heme-deficient mutants that had additional nutritional requirements (such as *cysG* in the model of Akutsu et al. [1]) would not have been recovered.

The hypothesis predicts that *cysG* mutants should be unable to synthesize heme anaerobically and should therefore require heme supplementation for anaerobic respiration and for aerobic respiration in a *hemF* mutant background. Anaerobic respiration was tested on minimal glycerol agar containing nitrate as the electron acceptor and cystine (Table 1). A *cysG*::MudJ insertion mutant (TE735) grew without supplements on this medium. Strain TE3154 (*hemA cysG*) grew on medium supplemented with either heme or the heme precursor δ-aminolevulinic acid (ALA), whereas TE2038 (*hemA hemN*) grew only if heme was provided. Thus, in contrast to *hemN* strains, *cysG* mutants do not display a requirement for heme during anaerobic growth, indicating that CysG-catalyzed uroporphyrinogen III methylation is not needed for anaerobic heme synthesis. No growth was seen even for the wild-type strain if nitrate was omitted, confirming that the incubation was anaerobic.

TABLE 1. Anaerobic growth on nitrate of *S. typhimurium* mutants

Strain	Relevant genotype[a]	Growth with the following supplement(s) to minimal cystine-glycerol-nitrate medium[b]:			
		None	ALA	Heme	Heme + ALA
LT-2	Wild type	+	+	+	+
TE735	*cysG*	+	+	+	+
TE1295	*hemA*	−	+	+	+
TE3154	*hemA cysG*	−	+	+	+
TE2038	*hemA hemN*	−	−	+	+
TE2720	*hemN*	−	−	+	+

[a] Complete genotypes are as follows: LT-2, *S. typhimurium* wild type; TE735, *cysG1573*::MudJ; TE1295, *hemA60 env-53*; TE3154, *hemA60 env-53 cysG1573*::MudJ; TE2038, *hemA60 env-53 hemN704*::MudJ; and TE2720, *env-53 hemN704*::MudJ.

[b] The minimal medium was NCE agar (3) containing 0.2% (wt/vol) glycerol as the carbon and energy source, 40 mM KNO_3 as the electron acceptor, and 150 μM L-cystine. Additions were made by spreading 0.1 ml of 1 mM ALA and/or 0.2 ml of 4-mg/ml hematin as indicated. Hematin was prepared as described previously (12). The anaerobic atmosphere was provided in a sealed jar by the GasPak Plus reagent (BBL Microbiological Systems) and monitored with a methylene blue indicator strip.

The Hem phenotype of *hemF cysG* double mutants was also determined during aerobic growth by comparing the abilities of TE735 (*cysG*::MudJ) and TE2036 (*hemN*::MudJ) to act as recipients in a transductional cross using a P22 donor lysate grown on TE3005 (*hemF*::Tn*10d*-Tet). Tet^r (*hemF* mutant) transductants were obtained with the *cysG* recipient but not with the *hemN* recipient, whereas both strains were efficient recipients when the donor carried an unrelated Tn*10d*-Tet insertion. Thus, unlike *hemF hemN* double mutants, *hemF cysG* double mutants are apparently not heme auxotrophs.

This conclusion was confirmed by constructing a *hemF cysG* double mutant under conditions permissive for heme auxotrophs, using as the recipient strain TE3154 (*hemA cysG*) and performing the transduction in the presence of heme and cystine. The resulting triple mutant strain, TE5773 (*hemA cysG hemF*), was able to grow aerobically on minimal glycerol agar

TABLE 2. Aerobic growth of *S. typhimurium* mutants

Strain	Relevant genotype[a]	Growth with the following supplement(s) to minimal glycerol medium[b]:			
		None	ALA	Cystine	Cystine + ALA
TE1295	*hemA*	−	+	−	+
TE5772	*hemA hemF*	−	+	−	+
TE3154	*hemA cysG*	−	−	−	+
TE5773	*hemA cysG hemF*	−	−	−	+

[a] Complete genotypes are as follows: TE5772, *hemA60 env-53 hemF707*::Tn*10d*-Tet; and TE5773, *hemA60 env-53 cysG1573*::MudJ *hemF707*::Tn*10d*-Tet (for others, see Table 1, footnote *a*).

[b] The minimal medium was NCE agar (3) containing 0.2% (wt/vol) glycerol as the carbon and energy source. Additions were made by spreading 0.1 ml of 1 mM ALA and/or 0.2 ml of 30 mM L-cystine as indicated.

if cystine and ALA were provided (Table 2). We conclude that *hemF cysG* double mutants are not heme auxotrophs, as they do not require heme supplementation for aerobic respiration.

The *cysG1573*::MudJ insertion used in these experiments has been shown previously to block cobalamin biosynthesis (i.e., it is defective in methylation of uroporphyrinogen III and cannot form precorrin-2). This insertion is also the most promoter proximal of all those mapped by Goldman and Roth (8), which makes it unlikely to retain residual functions of the other domains (precorrin-2 oxidase and sirohydrochlorin ferrochelatase) of this multifunctional protein (20, 23).

Conclusions. No biochemical or genetic evidence was found to support the hypothesis that the incorporation of methionine-methyl groups or the activity of *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase is required for the anaerobic biosynthesis of protoporphyrin IX-based tetrapyrroles in the organisms studied, which include a strict anaerobe as well as facultative anaerobes. Thus, the novel results of Akutsu et al. (1), which demonstrated the incorporation of methionine-methyl groups at positions 1 and 3 on the heme groups of cytochrome c_3 in *D. vulgaris*, do not appear to indicate a general anaerobic route to protoporphyrinogen IX that involves precorrin-2 rather than coproporphyrinogen III as an intermediate. Finally, although it seems possible that protoporphyrin IX-based heme *c* groups of cytochrome c_3 are formed by an alternate route in some strict anaerobes such as *D. vulgaris*, it would be useful to redetermine the structures of these hemes to verify that they are indeed heme *c*, rather than some other heme, such as heme d_1, that contains two methionine-derived methyl groups (28) and is probably derived from precorrin-2.

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