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Evidence for an Atypical Tetrapyrrole Biosynthetic Pathway in *Desulfovibrio*: Back Conversion of Sirohydrochlorin into Porphyrins

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Isotope labeling experiments performed on *Desulfovibrio vulgaris* have determined that two methyl groups of heme c and its precursors coprophyrin III and protoporphyrin IX come from the *S*-methyl group of methionine and not from the expected source which would be C2 of 5-aminolevulinic acid. *In vitro* biosynthetic experiments with broken-cell extracts led to the isolation of small quantities of novel new tetrapyrrole intermediates which could be structurally identified by visible spectroscopy and mass spectrometry. These intermediates suggest that sirohydrochlorin is decarboxylated at pyrrole rings C and D and deacteylated at rings A and B to produce coproporphyrin.

INTRODUCTION

The biosynthesis of common heme has been considered to be well established and is briefly depicted in Scheme **1.** A nonstandard compound designation system has been employed in this scheme. Compounds are designated according to the nominal integer mass observed for the protonated molecular ion (as observed in liquid secondary ion mass spectrometry (LSIMS[2])) for the oxidized, methyl ester derivative as would be obtained upon final isolation of the intermediate. Tetrapyrrole biosynthetic transformations take place on carboxylic acid-containing porphyrinogens or other relatively reduced states and structures are drawn in Scheme **1** reflecting this fact. However, these are air sensitive compounds and impractical to isolate as such. Purification and spectral characterization is easiest to perform after methyl esterification. Reactions leading to C-methylation (plus 16), decarboxylation (minus 58 reflected as the methyl ester derivative), and deacetylation (minus 74 reflected as the methyl ester derivative) play a prominent role in the following discussion, and the nonstandard designation scheme helps to remind the reader of the origins of the compound in question.

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[2] Abbreviations used: LSIMS, liquid secondary ion mass spectrometry; uro'gen III, uroporphyrinogen III; copro'gen III, coproporphyrinogen III; PPIX, protoporphyrinogen IX; SAM, S-adenosylmethionine; SUMT, *S*-adenosylmethhionine to uroporphyrinogen methyl transferase; FAB, fast atom bombardment mass spectrometry; ATCC, American Type Culture Collection; HPLC, high performance liquid chromatography.

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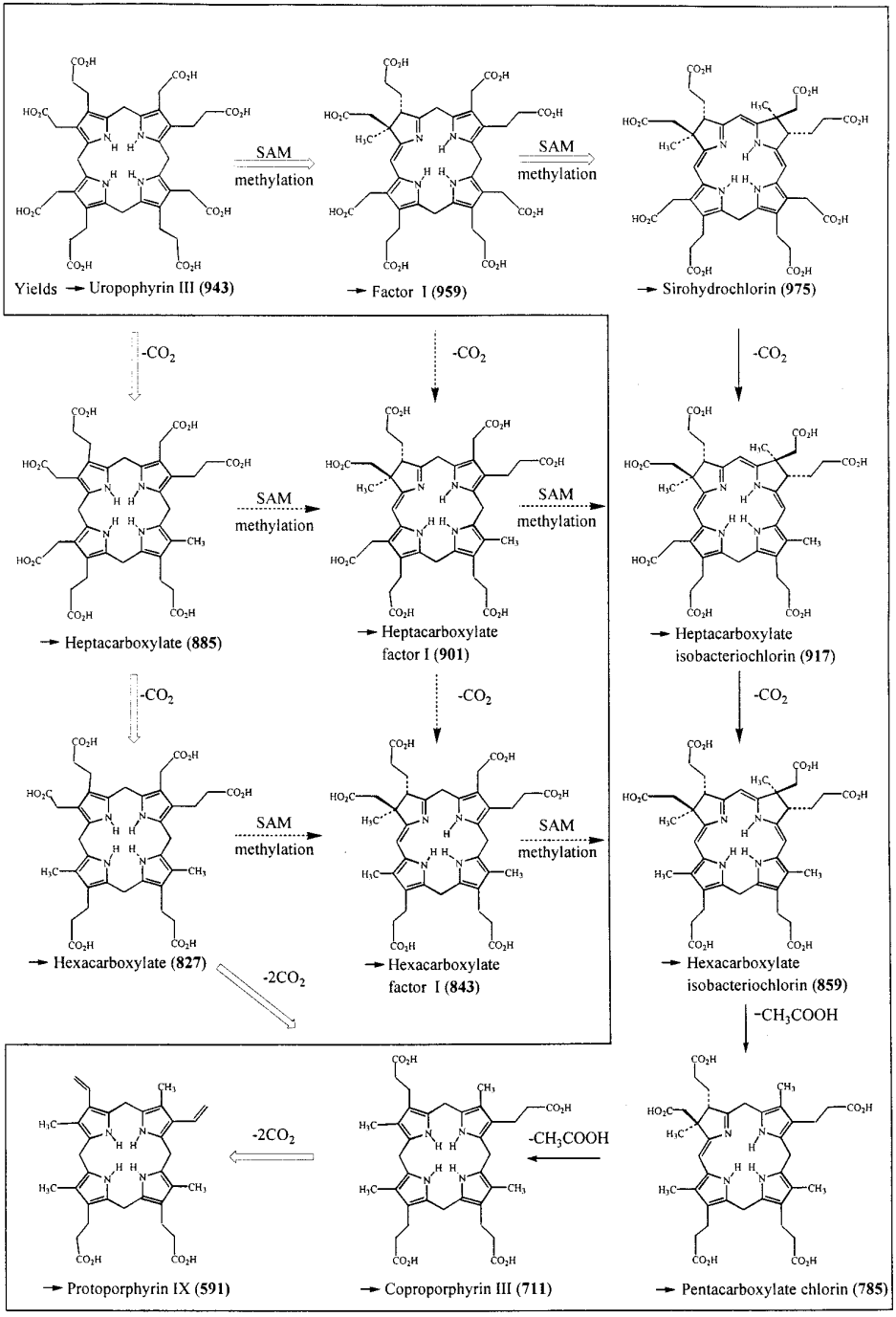
In the commonly expected route, uroporphyrinogen III, the ubiquitous tetrapyrrole precursor (uro'gen III, compound **943**), is successively decarboxylated at acetate substituents to coproporphyrinogen III (copro'gen, **711**). The two northern propionates (rings A and B) are then oxidatively decarboxylated to produce the two vinyl substituents of protoporphyrinogen IX (PPIX, **591**). Oxidation of the macrocycle and iron insertion produce heme b. Heme c results from the addition of cysteinyl-SH across the two vinyl groups to produce thioether bonds to polypeptide. Siroheme arises from a branching of this route. *S*-adenosylmethionine (SAM) to uro'gen methyl transferase (SUMT) C-methylates at C2 and C7 to produce the partially saturated tetrapyrrole dihydrosirohydrochlorin (**975**). This is a precursor on the route to vitamin B_{12}, but oxidation and iron insertion also produce siroheme. The C-methylation reactions have been considered as a fundamental branch point between the PPIX and sirohydrochlorin pathways. Akutsu *et al.* (*1*) reported that two northern methyl groups of heme c in the tetraheme cytochrome c3 from *Desulfovibrio vulgaris* arose from isotopically labeled methionine fed to the bacterial culture. This was a startling observation, since, by the commonly accepted route to heme c via PPIX, these methyl groups should arise from decarboxylation of acetates on uro'gen (**943**), which in turn have no connection to methionine. The observations suggested a mixing of the previously considered distinct PPIX and sirohydrochlorin branches. Their evidence was indirect in that it was based upon a decrease in intensity of hyperfine-shifted methyl resonances in the NMR spectrum of a complicated, multiheme paramagnetic protein (MW 13 kDa), interpreted as due to partial deuteration. The implications for heme biosynthesis are profound, so additional direct evidence has been sought for the biosynthetic route to heme c in *Desulfovibrio.*

EXPERIMENTAL

Liquid secondary ion mass spectrometry (LSIMS, also known as FAB) was performed on a VG Autospec spectrometer. The matrix for tetrapyrrole methyl esters was normally thioglycerol containing 1% trifluoroacetic acid, although in some cases "magic bullet" (1:1 thioglycerol:dithioerythreitol) or nitrobenzyl alcohol was also employed. In our experience these matrices invariably produce the $(M + H)^+$ ion as the dominant peak.

^{2}H- or ^{13}C-labeled methionine (99+%) was purchased from Aldrich Chemical

SCHEME 1. Possible biosynthetic routes in *Desulfovibrio vulgaris.* The structures are drawn as porphyrinogens or other reduced forms since this is the oxidation state at which the transformations are believed to occur. However, the trivial names correspond to the more oxidized forms that were subsequently isolated. The compound numbers in bold correspond to the observed molecular ion in mass spectrometry as $(M + H)^+$ for the methyl ester derivative. The open arrows represent the common pathway in other organisms. The dotted arrows represent possible transformations that were not observed. The boxed portion represents the pathway proposed for *D. vulgaris.*


SAM
methylation
SAM
methylation
Yields → Uropophyrin III (943)
→ Factor I (959)
→ Sirohydrochlorin (975)
-CO2
-CO2
-CO2
SAM
methylation
SAM
methylation
→ Heptacarboxylate (885)
→ Heptacarboxylate factor I (901)
→ Heptacarboxylate isobacteriochlorin (917)
-CO2
-CO2
-CO2
SAM
methylation
SAM
methylation
→ Hexacarboxylate (827)
-2CO2
→ Hexacarboxylate factor I (843)
→ Hexacarboxylate isobacteriochlorin (859)
-CH3COOH
-2CO2
-CH3COOH
→ Protoporphyrin IX (591)
→ Coproporphyrin III (711)
→ Pentacarboxylate chlorin (785)

Co. and used in a minimal medium (*2*) at a final concentration of 1 mM. *D. vulgaris* (ATCC 29579) cells from 36-h cultures were harvested by centrifugation and suspended (1 g wet weight of cells per 4 mL) in 0.1 M trihydroxyaminomethane–HCl buffer, pH 7. Under a nitrogen purge and on ice, the cell suspension was disrupted by ultrasonication in bursts of 5 min for a total of 15 min so that the suspension temperature never exceeds 4°C. The suspension was lyophilized. The oily solid was suspended in deoxygenated methanol (20 mL per g wet cells) and further deoxygenated by cycles of vacuum and nitrogen purging. Under a nitrogen atmosphere anhydrous HCl gas was bubbled through the suspension for circa 10 s and the reaction flask then sealed to exclude air. After 24 h at room temperature, the reaction vessel was opened to the atmosphere and chloroform added (4:1 to the methanol). The mixture was neutralized with aqueous sodium carbonate, washed three times with water, and the organic layer dried on a rotary evaporator. It was redissolved in chloroform and chromatographed on a small column (1 × 15 cm) of silica gel (200–400 mesh) eluted with chloroform. This crude column is not sufficient for purifying individual tetrapyrrole esters, but is very helpful for removing other background compounds that bind very tightly to silica gel and interfere with high resolution HPLC. Siroamide methyl ester stays bound to the column during chloroform elution and is subsequently removed by elution with ethyl acetate. Tetrapyrrole ester derivatives were further purified by HPLC on 5-μm silica gel eluted with chloroform containing 0.025% pyridine. In some cases fractions had to be rechromatographed in other solvents systems and these will be discribed as needed. Tetrapyrroles were identified by chromatographic comparison to standards, mass spectrometry, and visible spectrophotometry and quantified by standard extinction coefficients.

The strong mineral acid esterification condition employed to the total extract was sufficient to break thioether bonds to cysteine in heme c-containing proteins. Many common cytochromes c when placed in strongly acid methanol at room temperature form an insoluble precipitate and do not release heme. However, it is the physical separation that is preventing acid attack. Slama *et al.* (*3*) have discussed the complex products formed by acid attack upon cytochrome c under refluxing conditions. *D. vulgaris* contains many c-type cytochromes and some portion of these are susceptible to heme c cleavage at room temperature, converting the thioether bridges to two O-methyl substituents ($CH(S–Cys)–CH_3$ to $–CH(OCH_3)–CH_3$) and generating a derivative tetrapyrrole that gives a protonated molecular ion of 655 after esterification. We also separated a fraction that gave an observed m/z of 623 for $(M + H)^+$. This could have arisen from either O-methylation of one vinyl on protoporphyrin or the loss of one methanol equivalent from compound **655,** leading to a tetrapyrrole with one O-methyl and one vinyl group and the expected m/z of 623. The ratio **623:655** is approximately 2:7.

An *in vitro* system was prepared by suspending cells (5 g wet weight) in Tris buffer at the desired pH of the experiment and disrupting by ultrasonication as described above. Since nothing is known about whether tetrapyrrole biosynthesis enzymes in *Desulfovibrio* are soluble or membrane-associated, cell wall and membrane fragments were not removed and the resultant suspension is termed a broken cell extract. SAM *p*-toluene sulfonate salt (20 mg) and NAD^+ (20 mg) were added

from concentrated, deoxygenated stock solutions. Uro'gen III was prepared from uroporphyrin III dihydrochloride (Porphyrin Products, Logan, UT) by Na–Hg amalgam reduction in 1% aqueous ammonium hydroxide, which was neutralized to the pH of the experiment prior to addition by phosphoric acid. In different experiments the amount of uro'gen added ranged from 0.1 to 5 μmol. The reaction mixture was incubated anaerobically for 14 h at 37°C and then lyophilized. Ester derivatives were prepared and purified as described above.

The amount of recovered sirohydrochlorin methyl ester from the broken cell extract experiments corresponded to 65–70% of the starting uroporphyrin. While not quantitative, it represents a high rate of conversion. From control experiments it is known that only 60–70% of exogenously supplied porphyrin can be finally recovered from the broken cell extracts. Handling and chemical losses occur during reduction to porphyrinogens, reoxidation, and esterification and workup. A major source of loss is nonspecific binding, aggregation, and physical occlusion to the complicated broken cell suspension. The system produces authentic sirohydrochlorin and its epimers at C3 and C8 which were identified by proton NMR (*4*). Relative amounts were variable but approximately 2:1:1.

Factor I (compound **959**) was best resolved from other tetrapyrroles by silica gel HPLC in 10% hexane:90% chloroform with 0.025% pyridine where it eluted with capacity factor 11.7. The yield at pH 7.6 was 54 nmol from 2 μmol of starting uro'gen III. The ester derivatives **917** and **859** were less polar than sirohydrochlorin ester (**975**) or Factor I (**959**) and eluted from silica gel HPLC (chloroform with 0.025% pyridine) at capacity factors 4.5 (**917**) and 3.0 (**859**) and respective yields of 22 nmol (**917**) and 41 nmol (**859**) from 2 μmol of starting uro'gen III. In this solvent system, compound **785** elutes close to the void volume and is contaminated with coproporphyrin (**711**) and PPIX (**591**). Therefore material between capacity factors of 0 and 2.2 was pooled and rechromatographed in 30% hexane:70% chloroform with 0.025% pyridine. A peak at capacity factor 2.8 was shown by LSIMS to be **785** (56 nmol from 2 μmol uro'gen III). There were minor peaks in these chromatograms that LSIMS showed to be isomers of **917, 859,** and **785.** Isomers are possible because of the chiral centers present and/or a preference for which pyrrole has been affected. The differences in yields for these intermediates is likely due to the snapshot nature of the *in vitro* experiment. During the 14-h incubation, transformations have not gone to completion. Reaction progress is likely competing with loss of enzymatic activity. The rates of accumulation and disappearance of a given intermediate will depend upon precise substrate binding and enzymatic turnover parameters, and these can vary for different intermediates.

RESULTS AND DISCUSSION

Total endogenous tetrapyrroles were isolated from *D. vulgaris* grown on a simple medium supplemented with 1 mM methionine isotopically labeled at the *S*-methyl group in separate experiments with either [$^{13}CH_3$] or [C^2H_3]. It was possible to isolate the methyl esters of coproporphyrin (**711,** 1 nmol/g wet weight of cells), PPIX (**591,** 2 nmol/g), heme c derivatives (CH(S–Cys)–CH_3 to –CH(OCH_3)–CH_3

or $-CH=CH_2$, **655** and **623,** 9 nmol/g), sirohydrochlorin (**975,** 5 nmol/g), and siroamide (**960,** 60 nmol/g), the amidated form of sirohydrochlorin commonly found in *Desulfovibrio* (*5*). Exact mass determinations by high resolution liquid secondary ion mass spectrometry indicated that sirohydrochlorin had incorporated two equivalents of label from methionine methyl (*Anal.* Calcd for $C_{50}H_{57}[^2H_6]N_4O_{16}$ as $(M + H)^+$: 981.462; Found: 981.461), as expected for two successive C-methylations via SUMT. The surprising result was that coproporphyrin (*Anal.* Calcd for $C_{38}[^{13}C_2]H_{47}N_4O_8$: 713.346; Found: 713.346), protoporphyrin (*Anal.* Calcd for $C_{36}H_{33}[^2H_6]N_4O_4$: 597.335; Found: 597.339) and the heme c derivative (*Anal.* Calcd for $C_{36}[^{13}C_2]H_{47}N_4O_6$: 657.356; Found: 657.356) had also incorporated two equivalents of label (Fig. 1). These direct labeling experiments support the original work of Akutsu *et al.* (*1*) and indicate that two but only two of the four ring methyls in common porphyrins originate from methionine methyl.

Because of their low steady-state concentrations it is not possible to isolate all tetrapyrrole intermediates from viable cells. This limitation can be overcome in part by *in vitro* biosynthesis using broken cell extracts supplied with exogenous uro'gen and SAM. In a series of experiments, only 1–2.6% of the added uro'gen was recovered as the uroporphyrin ester **943,** indicating an efficient *in vitro* conversion of uro'gen. Essentially endogenous levels of siroamide **960,** coproporphyrin **711,** PPIX **591,** and heme c derivatives **623** and **655** were recovered, indicating poor conversion efficiency to these later products by the broken cell system. At pH values of 8.2 or above, the system produced only significant amounts of sirohydrochlorin **975** and its epimers, indicating a high *in vitro* activity for SUMT. The decarboxylase enzyme in chicken erythrocytes has been reported to be sensitive to pH (*6*). It appears that this is also the case in *D. vulgaris*, because, at lower pH values, the yield of **975** decreased and additional tetrapyrroles appeared. At pH 7.6 these included compounds **959** (known as Factor I), **917, 859,** and **785.**

Factor I (compound **959**) arises from a single C-methylation of uro'gen. It has been isolated and characterized previously (*7*). It was firmly identified from its characteristic chlorin-type visible spectrum, exact mass (*Anal.* Calcd for $C_{49}H_{59}N_4O_{16}$: 959.392; Found: 959.390), and its NMR spectrum which matched the previous report (*7*).

The visible spectra of **917** and **859** were highly typical of isobacteriochlorins and matched that of sirohydrochlorin ester closely except for a hypsochromic shift of circa 1 nm. Exact masses confirmed their identification (*Anal.* Calcd for $C_{48}H_{61}N_4O_{14}$: 917.418; Found: 917.414. Calcd for $C_{46}H_{59}N_4O_{12}$: 859.413; Found: 859.412). Note that for **917** two isomers are possible depending upon which pyrrole has been decarboxylated. No information is available regarding isomer or isomers present. For simplicity, only one is shown in Scheme **1.** Epimers are also possible at the chiral centers.

Compound **785** had a typical chlorin-type visible spectrum (wavelength (relative intensity): 393 nm (100), 497 nm (12), 645 nm (21)). Exact mass determination (obs. 785.377, (*Anal.* Calcd for $C_{43}H_{53}N_4O_{10}$: 785.376; Found: 785.377) confirmed the structure but does not establish the isomer(s) present.

By pooling samples identified by UV–visible and mass spectrometry over a period of time and by rechromatography via HPLC on silica, it was possible to obtain