L-Methionine Methyl is Specifically Incorporated into the C-2 and C-7 Positions of the Porphyrin of Cytochrome c_3 in a Strictly Anaerobic Bacterium, *Desulfovibrio vulgaris*

Hideo Akutsu,*,† Jang-Su Park,†,‡ and Seiyo Sano§

Department of Bioengineering
Faculty of Engineering
Yokohama National University
Hodogaya-ku, Yokohama 240, Japan
Shiga University of Medical Science
Ohtsu, Shiga 520-21, Japan

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Cytochrome c_3 is an electron transport protein with four *c*-type hemes found in sulfate-reducing bacteria. The crystal structure of this protein has been established for *Desulfovibrio desulfricans* Norway,[1] *Desulfovibrio vulgaris* Miyazaki F,[2] and *D. vulgaris* Hildenborough.[3] We have succeeded in estimating 32 microscopic redox potentials of cytochrome c_3 from *D. vulgaris* Miyazaki F by use of NMR.[4] In order to establish the relationship between these microscopic redox potentials and the hemes in the crystal structure, we have carried out selective labeling of the phenylalanine, tyrosine, histidine, and methionine residues on this protein.[5-7] During these experiments, we made a surprising observation. Namely, the deuterated methyl group of methionine-*methyl-d*$_3$ in the medium was incorporated not only into the methionine residues of cytochrome c_3 but also into particular heme methyl groups of this protein. This suggests the existence of a new porphyrin biosynthetic pathway.

A sulfate-reducing bacterium, *D. vulgaris* Miyazaki F, was cultured in either medium C[8] or a minimal medium containing a mixture of amino acids.[5] To perform selective deuteration of cytochrome c_3, L-methionine in the minimal medium was replaced with L-methionine-*methyl-d*$_3$ (^{2}H; 98% Cambridge Isotope Laboratories). Cytochrome c_3 was purified as reported elsewhere.[5]

^{1}H NMR spectra (400 MHz) of nonlabeled and deuterated cytochrome c_3 in the fully oxidized state for the region from 6 to 32 ppm are presented in Figure 1. Since the hemes are paramagnetic in the oxidized state (low spin), the signals of the heme groups are scattered in a wide range. The strong peaks with three-proton intensity in the low-field region can be attributed to the heme methyl groups. Most of them have now been assigned to specific methyl groups of each heme by us.[9] In the spectrum of the deuterated cytochrome c_3, a significant decrease in the signal intensity was observed for eight methyl signals of four hemes, which are indicated by broken lines in the figure. The assignment given at the top of the figure shows that the diminished signals belong to the C-2 and C-7 methyl groups of the porphyrin. There was no change, however, in other signals, including those of the C-12 and C-18 methyl groups of the porphyrin. Thus, it can be concluded that the C-2 and C-7 methyl groups of the porphyrin of cytochrome c_3 are transferred from the ϵ-methyl group of L-methionine, presumably via *S*-adenosylmethionine, during biosynthesis.

In the known porphyrin biosynthetic pathway, all four methyl groups of protoporphyrin IX, which is the precursor of heme *c*, are transformed from acetate groups by uroporphyrinogen decarboxylase.[10] This pathway cannot explain the incorporation of methyl groups into the C-2 and C-7 positions from methionine. Since it is well established that the chemical structure of the hemes of cytochrome c_3 is identical with that of the *c*-type hemes in mitochondria,[2] our finding is strong evidence for the existence of an alternative pathway for the biosynthesis of *c*-type hemes in *D. vulgaris* Miyazaki F, suggesting that a previously unknown pathway of anaerobic porphyrin biosynthesis exists in *D. vulgaris*.

Although methyl transfer from methionine has not been reported yet for mammalian *c*-type hemes, *S*-adenosylmethionine plays an important role in the biosynthesis of vitamin B_{12}.[11] It is known that two methyl groups are transferred from *S*-adenosylmethionine to the C-2 and C-7 positions of uroporphyrinogen III, leading to sirohydrochlorin (Figure 2).[11] Iron sirohydrochlorin (called siroheme) is the prosthetic group of sulfite reductase (desulfoviridin),[12] which is abundant in the cytoplasm of sulfate-reducing bacteria. A probable intermediate from uroporphyrinogen III could be formed through methylation at the C-2 and C-7 positions, followed by deacetylation of the acetate groups at the same positions. The accumulation of a large amount of sirohydrochlorin in *D. vulgaris* Miyazaki F in the presence of δ-aminolevulinic acid has been observed (Oiso *et al.*, unpublished data). This pathway could also be involved in the biosynthesis of heme d_1. The chemical structure of heme d_1 was determined to be that of a dione heme by Wu and Chang.[13] It can be obtained by replacing the propionate groups at the C-2 and C-8 positions of sirohydrochlorin through pinacol rearrangement with oxygen and by introduction of a double bond into the propionate group at the C-13 position. Thus, sirohydrochlorin could be a common precursor of hemes of *c* and d_1 in anaerobic bacteria.

In the known pathway, molecular oxygen is needed for the oxidative decarboxylation of coproporphyrinogen III.[14-17] Molecular oxygen, however, would not be available to strict anaerobes such as sulfate-reducing bacteria. A number of groups have carried out investigations to clarify the anaerobic biosynthesis of porphyrin.[18-21] No evidence for a new pathway, however, has been found so far. For the first time, our work has provided clear

† Yokohama National University.
‡ Present address: Department of Chemistry, Pusan National University, #30, Changjum-Dong, Kumjeon-Ku, Pusan 609-735, Korea.
§ Shiga University of Medical Science.

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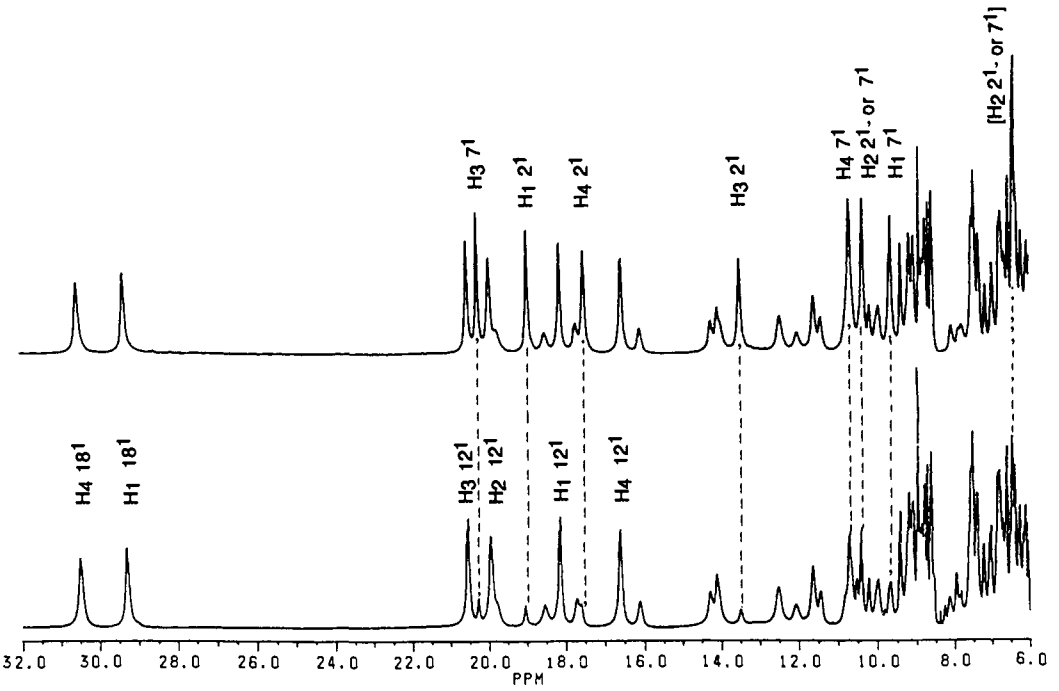

Figure 1. ^1H NMR spectra (400 MHz) of ferric cytochrome c_3 from *D. vulgaris* Miyazaki F in 20 mM phosphate buffer (pH 7.0) at 30 °C. Top: Nonlabeled protein. Bottom: Deuterated protein obtained from cells cultured in a minimal medium with L-methionine-*methyl-d*$_3$. The signals affected by deuteration are indicated by broken lines. The established assignments are shown in the figure for the heme methyl signals of interest. Heme *i* (numbering according to the sequence) is denoted by H_i. The atom-numbering scheme for heme *c* is given in Figure 2. The assignment performed in this work is given in square brackets. The spectra were measured with a Bruker AM-400 NMR spectrometer. Chemical shifts are presented relative to the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

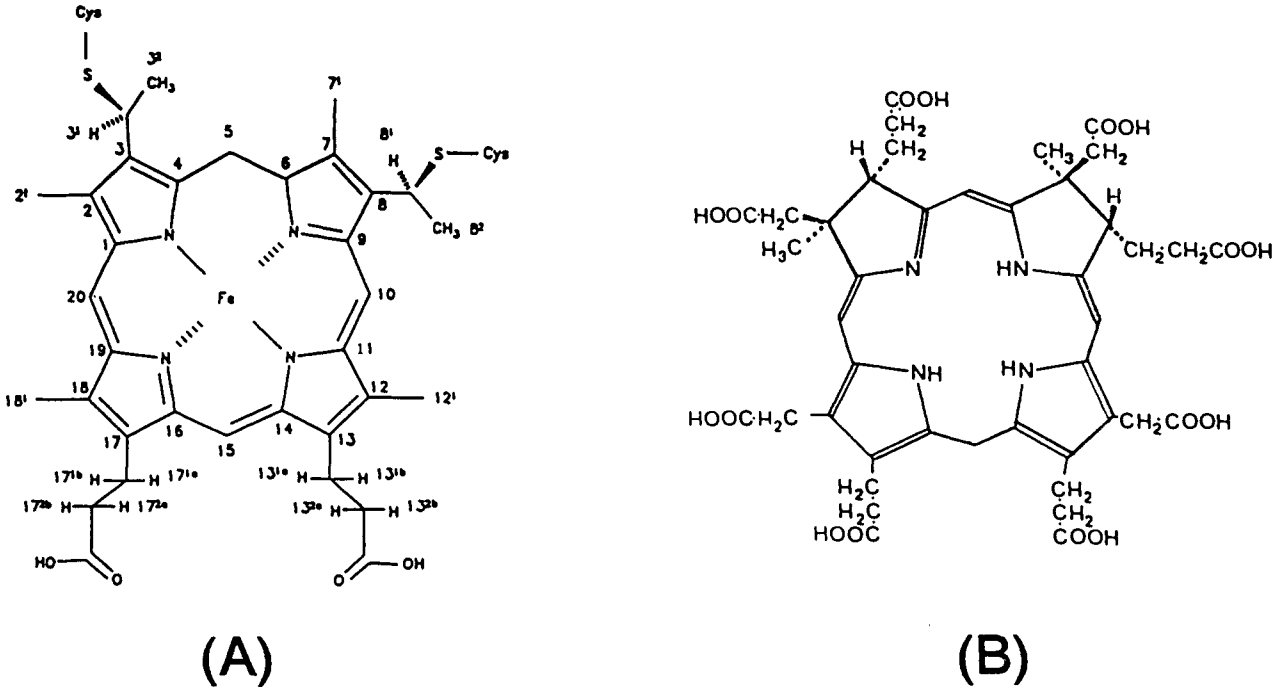

Figure 2. IUPAC–IUB atom-numbering scheme for heme *c* (A) and the chemical structure of sirohydrochlorin (B).

evidence for the existence of a new porphyrin biosynthetic pathway, which presumably includes sirohydrochlorin as an intermediate. Investigation of the new biosynthetic pathway would also contribute to elucidation of the evolution of porphyrin biosynthesis.