Studies on the Biosynthesis of Corrinoids and Porphyrinoids. X.[1] Biosynthetic Studies of Bacteriochlorophyll-a in *Rhodopseudomonas spheroides*: Incorporation of ^{2}H-, ^{13}C- and ^{15}N-Labeled Substrates and Origin of the Hydrogen Atoms

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The biosynthetic pathway of bacteriochlorophyll-a in *Rhodopseudomonas spheroides* was investigated by administration of [2-^{13}C]glycine, L-[methyl-^{13}C]methionine, L-[1-^{13}C]glutamic acid and ^{13}C- or ^{15}N-labeled δ-aminolevulinic acid (ALA). The origin of the hydrogen atoms was studied by following the incorporation into bacteriochlorophyll of ^{13}C-labeled ALA in a medium containing 50% D_2O and that of ^{2}H-, ^{13}C-labeled ALA. The labeled bacteriochlorophylls were converted into more stable methyl bacteriopheophorbides, which were subjected to ^{13}C-NMR analyses to obtain information about the biosynthetic origin of the hydrogen atoms. The use of ^{13}C-enriched bacteriochlorophylls and methyl bacteriopheophorbides allowed us to make ^{13}C-NMR signal assignments for all carbons of the porphyrin nucleus.

Key words bacteriochlorophyll; methyl bacteriopheophorbide; *Rhodopseudomonas spheroides*; biosynthesis; δ-aminolevulinic acid

We have investigated the biosynthetic pathway of tetrapyrrole compounds such as vitamin B_{12}[2] and chlorophyll.[3] Knowledge of the formation of the carbon backbone is almost complete. First, two molecules of δ-aminolevulinic acid (ALA) condense to form porphobilinogen (PBG), which is converted to uroporphyrinogen III. From this intermediate, vitamin B_{12} and chlorophyll are derived.[4] In the case of the biosynthesis of chlorophyll, two pathways exist for the formation of ALA. The first is the Shemin pathway,[5] in which ALA is formed by the condensation of glycine and succinyl-CoA. The second pathway is the C-5 pathway,[6] in which ALA is derived from all the carbons of glutamate or 2-oxoglutarate.

In 1985 Oh-hama *et al.*[7] reported that biosynthesis of bacteriochlorophyll (Bchl) in *Rhodopseudomonas spheroides* S proceeds through ALA synthesized *via* the Shemin pathway. Emery and Akhter[8] examined the origin of the oxygen atoms to confirm the mechanism of introduction of the esterifying alcohol. However, knowledge of the origin of hydrogen atoms is still fragmentary. We therefore planned to examine the origin of hydrogen of Bchl, by incorporating ^{13}C-labeled substrate in a medium containing 50% D_2O and by incorporating ^{2}H-, ^{13}C-labeled substrate into Bchl. The use of biosynthetically ^{13}C-labeled Bchls and their derivatives (methyl bacteriopheophorbide; MeBpheo) allowed us to make ^{13}C-NMR signal assignments.

Results and Discussion

After incubation of *R. spheroides* with [2-^{13}C]glycine, Bchl was isolated and subjected to ^{13}C-NMR analysis. The spectrum revealed ^{13}C enrichment at eight carbons, compared with the intensities of the natural abundance. The ^{13}C-enriched carbons would have been derived from [5-^{13}C]ALA. [5-^{13}C]ALA itself was subsequently incorporated into Bchl under identical conditions. The ^{13}C-NMR spectrum showed high enrichment of the same eight carbons (δ, α, β, γ, C-12, C-17, C-14, and C-16), whose signals were split into doublets owing to ^{13}C–^{13}C spin coupling with adjacent ^{13}C atoms, except for the δ carbon (Fig. 1a). Next, an incorporation experiment with L-[1-^{13}C]glutamic acid showed no enrichment at the eight carbons, which would be expected if the C-5 pathway were involved. The data showed that ALA was biosynthesized by ALA synthase *via* glycine through the Shemin pathway. These biosynthetic results are similar to those reported previously by Oh-hama *et al.*,[7] except that C-2 of glycine was also incorporated specifically into the methyl ester carbon (C-10b) of Bchl. In the [2-^{13}C]glycine feeding experiment, we did not detect an enriched signal of the C-10b methyl carbon. The methyl carbon is presumably derived from C-methylation *via* S-adenosylmethionine (SAM). When L-[methyl-^{13}C]methionine was incorporated into Bchl under identical conditions, the ^{13}C-NMR spectrum showed enrichment of the C-10b methyl carbon (52.11 ppm) (Fig. 1b).

Glycine can act as a one-carbon donor by carbon transfer to tetrahydrofolate, which can then transfer the carbon to homocysteine to form methionine, a versatile re-carbon donor. In this process the C-2 of glycine may be transformed into methylene of N^5,N^{10}-methylene-tetrahydrofolate either directly by glycine synthase or indirectly *via* the C-3 of serine by serine hydroxymethyltransferase. The results of our [2-^{13}C]glycine feeding experiment suggest that these enzymes are not active under our feeding conditions, and the label is preferentially incorporated into ALA by ALA synthase. The differences concerning methionine biosynthesis from the C-2 of glycine presumably reflect differences in feeding conditions or in the strains of bacteria used in the two experiments.

The above labeling experiments and the combination of four separate labeling experiments with [1-^{13}C]-, [2-^{13}C]-, [3-^{13}C]- and [4-^{13}C]ALA accounted for the origin of 35 of the 55 carbons in Bchl (the remaining twenty carbons are derived from phytol *via* mevalonic

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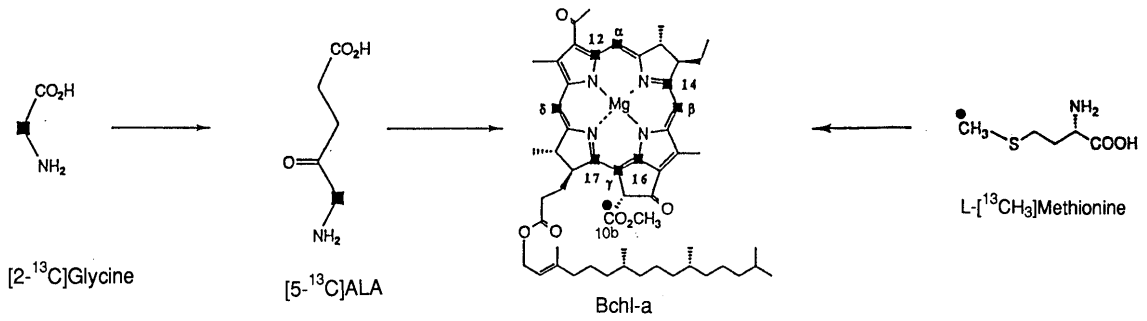

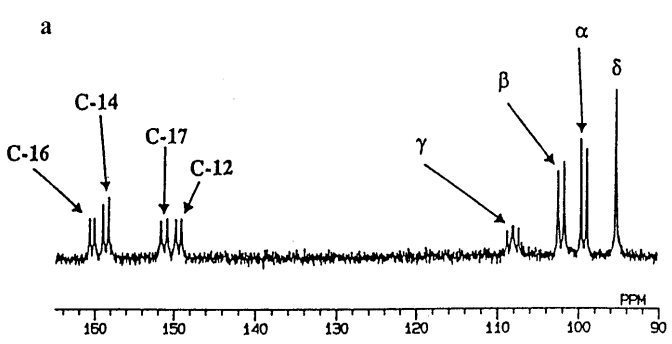

Fig. 1a.

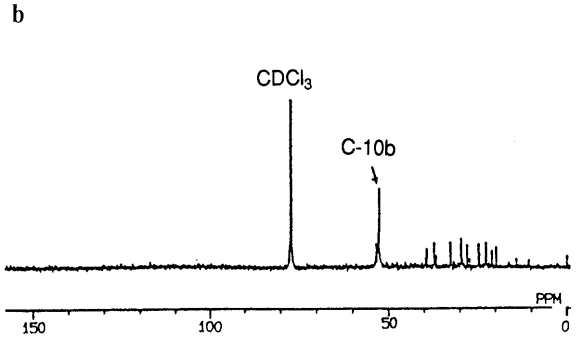

Fig. 1b.

Fig. 1. Chemical Structures of Labeled Bacteriochlorophyll-a and the Labeled Precursors
1a. Expanded ^{13}C-NMR Spectrum of Bacteriochlorophyll-a Enriched from [5-^{13}C]ALA
1b. ^{13}C-NMR Spectrum of Bacteriochlorophyll-a Enriched from L-[$^{13}CH_3$]Methionine

acid). These biosynthetically labeled Bchls allowed ^{13}C-NMR signal assignments to be made easily, because specific carbons are labeled with ^{13}C. We were able to make signal assignments for all the carbons of the porphyrin nucleus (Table 1). Most of the carbons were assigned by comparison with published data,[9-11] but the quaternary carbons C-1 and C-2 were reassigned in the present study.[9] These carbons can be distinguished by making use of the products from the feeding experiments with [3-^{13}C]ALA and [4-^{13}C]ALA, and we concluded that the resonances of C-1 (127.6 ppm) and C-2 (122.3 ppm) previously assigned should be interchanged as shown in Table 1. The ^{13}C-labeled Bchls were then converted into more stable methyl bacteriopheophorbides (MeBpheo) by treatment with 0.5% sulfuric acid in methanol. The ^{13}C-NMR data are shown in Table 2. The assignments were similar to those of previous reports.[11]

To clarify the origin of nitrogen atoms of Bchl-a, ^{15}N-ALA was fed to *R. spheroides* and Bchl-a was isolated, and converted to the corresponding MeBpheo for ^{15}N-NMR spectroscopy (Fig. 2). Four distinct signals were observed at 12.77, 18.42, 182.63, and 190.23 ppm (formamide = 0 ppm as an external reference). The chemical shifts of these peaks are in the range of amine and imine nitrogens.[12] As no ^{15}N-NMR signals were obtained on measurement of unlabeled MeBpheo, these four peaks are due to incorporated ^{15}N-ALA.

Studies on the origin of hydrogen atoms might provide useful information concerning the mechanisms of formation of biosynthetic intermediates. In order to determine the source of the hydrogens in Bchl-a, [2-^{13}C]ALA and [3-^{13}C]ALA in 50% D_2O medium and ^{2}H-, ^{13}C-labeled ALA were next used as precursors. The resulting Bchls were isolated and converted to the corresponding MeBpheos for ^{13}C-NMR. The ^{13}C-NMR spectra of MeBpheo derived from the feeding experiments with [2-^{13}C]ALA in 50% D_2O-containing medium and with [2-^{13}C, 2-$^{2}H_2$]ALA are shown in Fig. 3. In the [2-^{13}C, 2-$^{2}H_2$]ALA feeding experiment, the signals of C-4b, C-5a, C-1a, C-8a, C-3a, and C-7b were split into triplets and quintets owing to ^{2}H–^{13}C spin coupling and these signals were shifted upfield by 24.9—57.2 Hz owing to deuterium α-isotope effects. These coupled signals with α-shift clearly revealed the presence of two or one deuterium atoms in the C-4b, C-5a, C-1a, C-8a, and C-3a methyl groups and the C-7b methylene group. No deuterium was detected at C-2b or C-10, which are known to undergo prototropic exchange under acidic conditions. However, the product obtained from [2-^{13}C]ALA in 50% D_2O-containing medium did not give such ^{2}H–^{13}C spin coupling or clear deuterium α-isotope effect, but showed low incorporation at C-4b, C-5a, C-1a, C-8a and C-3a in comparison with the signal intensities of the control, suggesting that these methyl groups were deuterated or influenced by adjacent deuterium. To investigate this further, we applied broad-band deuterium and proton-decoupled ^{13}C-NMR (^{13}C-{^{1}H}{D}NMR) spectroscopy.[13] The expanded ^{13}C-{^{1}H}{D}NMR spectrum of [2-^{13}C, 2-$^{2}H_2$]ALA-derived MeBpheo showed two singlet signals at C-4b, C-5a, C-1a, C-8a, C-3a, and C-7b respectively, indicating the existence of two deuteriums (CHD_2) with an α-shift of 47.2—59.0 Hz upfield and one deuterium (CH_2D) with an α-shift of 23.6—29.5 Hz upfield (Fig. 4 and Table 3). These results exclude the possibility of the biosynthesis of Bchl-a from ALA involving complete loss of the deuterium atoms in [2-^{13}C, 2-$^{2}H_2$]ALA. The ^{13}C-{^{1}H}{D}NMR spectrum of MeBpheo derived from [2-^{13}C]ALA in 50% D_2O-containing medium clearly

Table 1. ^{13}C Chemical Shifts in Bacteriochlorophyll-a

Assignment	Natural		Feeding experiments					
Carbon No.	Solvent (a)	Solvent (b)	[1-^{13}C] ALA	[2-^{13}C] ALA	[3-^{13}C] ALA	[4-^{13}C] ALA	[5-^{13}C] ALA	L-[$^{13}CH_3$] Methionine
2a	199.92	200.01			200.07			
9	188.44	189.75			189.80			
7c	174.01	173.96	173.93					
10a	170.60	172.38	172.34					
18	168.75	168.17				168.15		
11	166.64	166.87				166.88		
16	160.37	160.64					160.85 (63.9)[c]	
14	158.62	158.69					158.77 (75.9)	
17	151.23	152.19					152.35 (81.4)	
15	150.44	150.74				150.71		
12	149.41	149.91					150.13 (71.6)	
13	149.07	149.57				149.45		
P3	143.50	143.06						
5	141.49	142.86			143.11			
6	136.78	137.49				137.48		
2	129.15	129.97				129.87		
1	125.13	124.25			124.22			
P2	116.77	119.45					109.50 (63.9, 81.4)	
γ	108.07	109.42					101.96 (75.9)	
β	102.13	101.89					99.53 (71.6)	
α	99.30	99.48					96.33	
δ	95.30	96.22						
10	64.51	66.06		65.99				
P1	61.88	61.71						
4	54.86	56.05				55.94		
10b	52.63	52.67						52.11
7	49.46	51.07				50.98		
8	49.07	50.03			50.03			
3	47.56	48.76			48.76			
P4	39.83	40.41						
P14	39.36	40.15						
P10	37.43	38.15						
P8	37.37	38.09						
P12	37.29	38.04						
P6	36.75	37.28						
P11	32.78	33.55						
P7	32.67	33.38						
2b	32.47	33.18		33.05				
7b	30.63	31.29		31.23				
4a	30.32	31.07			31.09			
7a	29.56	30.74			30.75			
P15	27.96	28.75						
P5	25.06	25.75						
P13	24.78	25.54						
P9	24.47	25.15						
3a	22.98	23.53		23.45				
8a	22.88	23.46		23.39				
P15a	22.62	23.00						
P16	22.62	22.92						
P7a	19.73	20.10						
P11a	19.66	20.04						
P3a	16.17	16.27						
1a	13.63	13.76		13.70				
5a	11.88	12.13		12.07				
4b	10.66	10.86		10.80				

Chemical shifts (ppm) are given downfield from TMS. *a*) $CDCl_3$. *b*) Acetone-d_6 : $CD_3OD = 4:1$. *c*) ^{13}C–^{13}C coupling constants in Hz.

showed one singlet signal at C-5a and C-1a, indicating the existence of one deuterium (CH_2D) with an α-shift of 25.1 Hz upfield. The signals of C-8a and C-3a corresponded to one deuterium (CH_2D) with an α-shift, but these signals overlapped each other (Table 4). These results indicated that one of the hydrogen atoms of each of the C-5a, C-1a, C-8a, and C-3a methyl groups is derived from water in the medium. On the other hand, C-4b shows deuterium with a shift of 11.8 Hz upfield; this is too small to be an α-shift, but may correspond to a β-deuterium isotope shift arising from the presence of ^2H at C-4a. Complementary and supporting data were obtained from the experiment with [3-^{13}C]ALA in 50% D_2O-containing medium, whose ^{13}C-{^1H}{D}NMR spectrum showed an

Table 2. ^{13}C Chemical Shifts in Methyl Bacteriopheophorbide-a

Assignment	Natural	Feeding experiments					
Carbon No.	Solvent $CDCl_3$	[1-^{13}C]ALA	[2-^{13}C]ALA	[3-^{13}C]ALA	[4-^{13}C]ALA	[5-^{13}C]ALA	Methyl ester
2a	199.15			199.15			
9	189.05			189.05			
7c	173.33	173.33					
18	171.05				171.05		
11	169.55				169.55		
10a	169.53	169.53					
12	163.67					163.63 (8.8, 74.8)[a]	
16	157.91					157.89 (10.3, 82.2)	
17	148.08					148.07 (66.1)	
15	139.07				139.07		
13	138.29				138.32		
14	136.83					136.82 (71.9)	
5	136.27			136.24			
6	133.29				133.29		
2	129.84				128.62		
1	121.37			121.34			
γ	108.00					107.99 (66.1, 82.2)	
α	99.64					99.60 (74.8)	
β	97.62					97.63 (10.3, 71.9)	
δ	95.78					95.77 (8.8)	
10	64.35		64.36				
4	54.96				54.96		
10b	52.80						52.80
7d	51.69						51.69
7	50.60				50.60		
8	49.69			49.69			
3	48.86			48.86			
2b	33.35		33.35				
7b	30.93		30.95				
4a	30.18			30.16			
7a	29.93			29.92			
3a	22.93		22.93				
8a	22.85		22.85				
1a	13.42		13.42				
5a	11.53		11.53				
4b	10.75		10.75				

Chemical shifts (ppm) are given downfield from TMS. *a*) ^{13}C–^{13}C coupling constants in Hz.

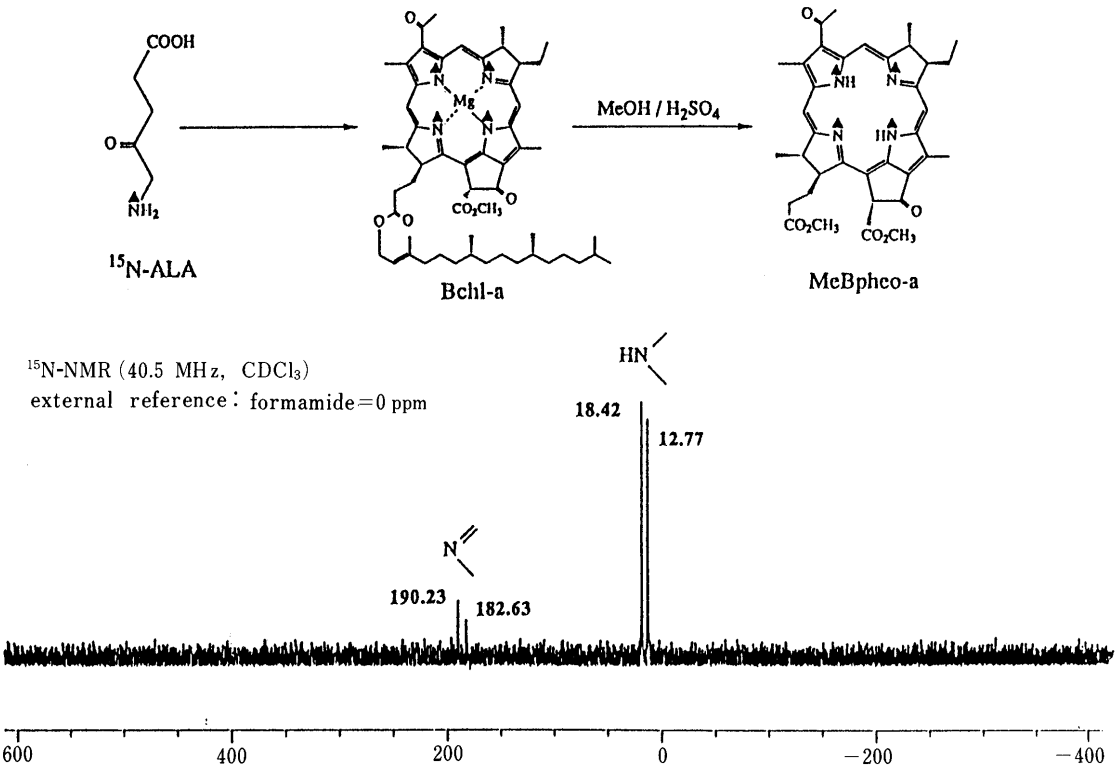

Fig. 2. ^{15}C-NMR Spectrum of Methyl Bacteriopheophorbide-a Enriched from ^{15}C-ALA

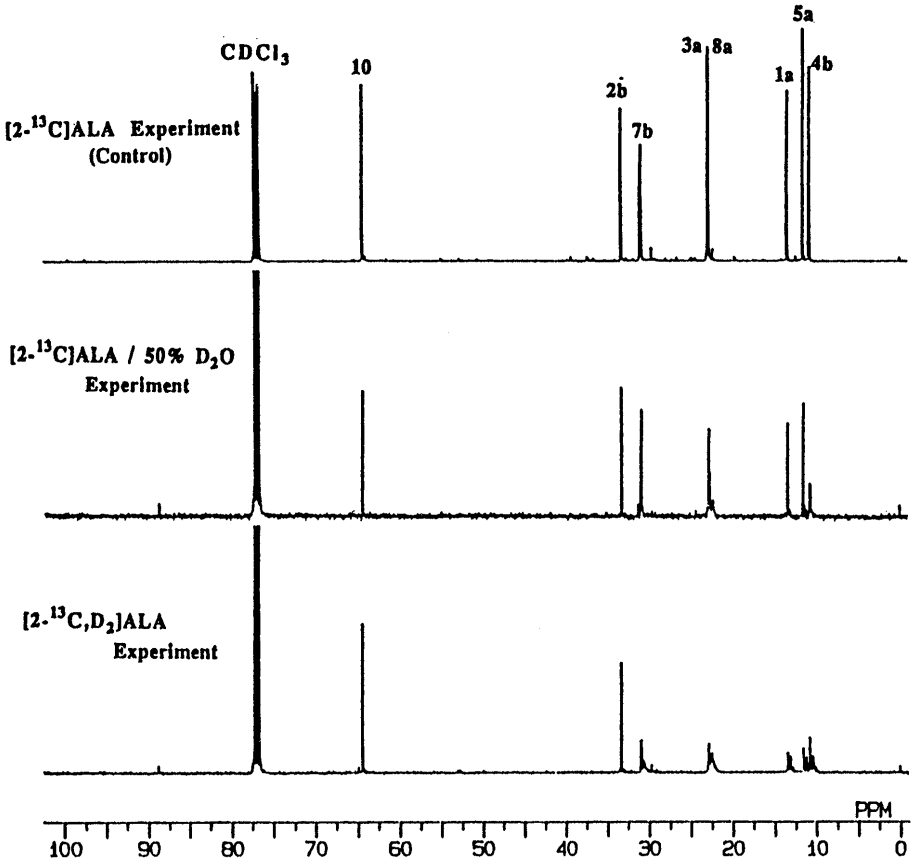

Fig. 3. Comparison of the ^{13}C-{^{1}H}NMR of [2-^{13}C]ALA/50% D_2O-derived Methyl Bacteriopheophorbide-a (B) and [2-^{13}C, D_2]ALA-derived Methyl Bacteriopheophorbide-a (C) with [2-^{13}C]ALA-derived (control) Methyl Bacteriopheophorbide-a (A)

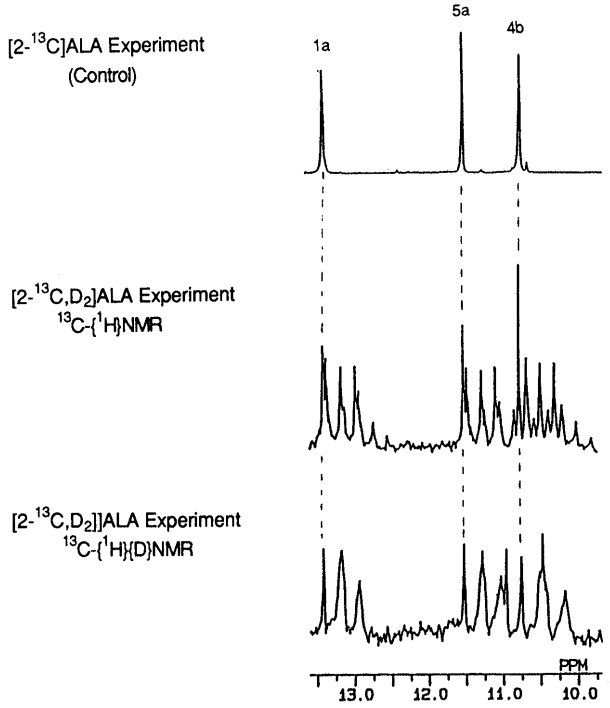

Fig. 4. Expanded ^{13}C-NMR Spectrum of Methyl Bacteriopheophorbide-a Enriched from [2-^{13}C, D_2]ALA. (A): Control (^{13}C-{^{1}H}NMR), (B): ^{13}C-{^{1}H}NMR, (C): ^{13}C-{^{1}H}{D}NMR

α-shifted resonance at C-4a. The signal due to C-4b in the spectrum of the product derived from [2-^{13}C]ALA in 50% D_2O-containing medium showed only a β-shifted res-

Table 3. Isotope Shifts of Labeled Methyl Bacteriopheophorbide-a Enriched from [2-^{13}C, D_2]ALA

Substrate	Carbon No.	Chemical shift (ppm)	α-Shift (Hz)	Species
[2-^{13}C, 2-D_2]ALA	4a	10.75	29.4	CDH
			58.9	CD_2
	5a	11.53	25.1	CDH
			48.7	CD_2
	1a	13.42	23.6	CDH
			47.2	CD_2
	8a	22.85	29.4	CDH
			57.5	CD_2
	3a	22.92	29.5	CDH
			59.0	CD_2
	7b	30.95	28.0	CDH
			Unclear	CD_2

Table 4. Isotope Shifts of Labeled Methyl Bacteriopheophorbide-a Enriched from [2-^{13}C]ALA/50% D_2O

Substrate	Carbon No.	Chemical shift (ppm)	α-Shift (Hz)	Species
[2-^{13}C] ALA/ 50% D_2O	4b	10.75	11.8 (β-shift)	C-CDH
	5a	11.53	25.1	CDH
	1a	13.42	25.1	CDH
	8a	22.85	25.4—38.3[a)]	CDH
	3a	22.92	25.4—38.3[a)]	CDH
	7b	30.95	Not detected	—

a) Overlapped each other.

onance and no α-shifted resonance. This indicates that C-4b was not deuterated and one hydrogen atom of the C-4a methylene group was deuterated from the medium. From these results, it is clear that two of the hydrogen atoms of the C-4b methyl group are retained in the biosynthetic process from ALA. We suggest that the other hydrogen is derived from hydride, generated from reduced nicotinamide adenine dinucleotide (NADH). These findings are consistent with the established mechanism,[14] including the reduction of the vinyl group of protoporphyrinogen IX, which is biosynthesized[4] from uroporphyrinogen III *via* ALA (Fig. 5).

Thus, we conclude that two of the hydrogen atoms of the C-5a, C-1a, C-8a and C-3a methyl groups are retained from ALA. The remaining one is derived from water in the medium. Both hydrogen atoms of the C-7b methylene group are retained from ALA (Fig. 6). These results strongly support the proposed reaction mechanism[15] of porphyrinogen carboxy-lyase, which catalyzes the decarboxylation of the four acetate side chains of uroporphyrinogen III (Fig. 7).

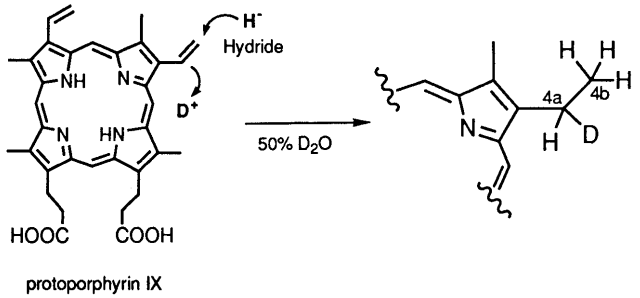

Fig. 5. Hypothetical Mechanism for Reduction of the Vinyl Group of Protoporphyrin IX in 50% D_2O

Experimental

Instruments ^{13}C-NMR, ^{13}C-{^{1}H}{D}NMR, and ^{15}N-NMR spectra were recorded on a JEOL GSX-400 spectrometer (100 or 40 MHz) in $CDCl_3$ or acetone-d_6 : CD_3OD (4 : 1) solution with tetramethylsilane (TMS) as an internal standard in the case of ^{13}C-NMR, and formamide (= 0 ppm) in the case of ^{15}N-NMR. The conditions were: spectral width 24,038.5 Hz; acquisition time 0.682 s; repetition delay 2.5 s; and 30° pulse.

Materials and Organism ^{13}C-Labeled ALAs and [2-^{13}C]glycine were synthesized by literature procedures[16] from [^{13}C]sodium acetate. L-[1-^{13}C]Glutamic acid was synthesized by literature procedures[3] from potassium [^{13}C]cyanide. L-[Methyl-^{13}C]methionine was synthesized by literature procedures[17] from [^{13}C]methyl iodide. [2-^{13}C, D_2]ALA was synthesized by literature procedures[16] from [2-^{13}C, D_3]sodium acetate. The strain used was *Rhodopseudomonas spheroides* IFO 12203.

Culture Conditions *Rhodopseudomonas spheroides* IFO 12203 was grown anaerobically in the light (100 W × 2) at 30 °C in a static culture, each flask holding 60 ml of an aqueous solution containing yeast extract (2 g), monosodium L-glutamate (2.9 g), DL-malic acid (2.7 g), KH_2PO_4 (500 mg), K_2HPO_4 (500 mg), $(NH_4)_2HPO_4$ (800 mg), $MgSO_4$ (100 mg), $CaCl_2$ (40 mg), nicotinic acid (1 mg), thiamine hydrochloride (1 mg), biotin (10 μg), $MnCl_2$ (630 μg), and $FeCl_3 \cdot 6H_2O$ (2.5 mg) per liter of distilled water at pH 6.8 (adjusted with KOH solution).

Incorporation of Labeled Precursor Fresh cells (2-d-cultured, *ca.*

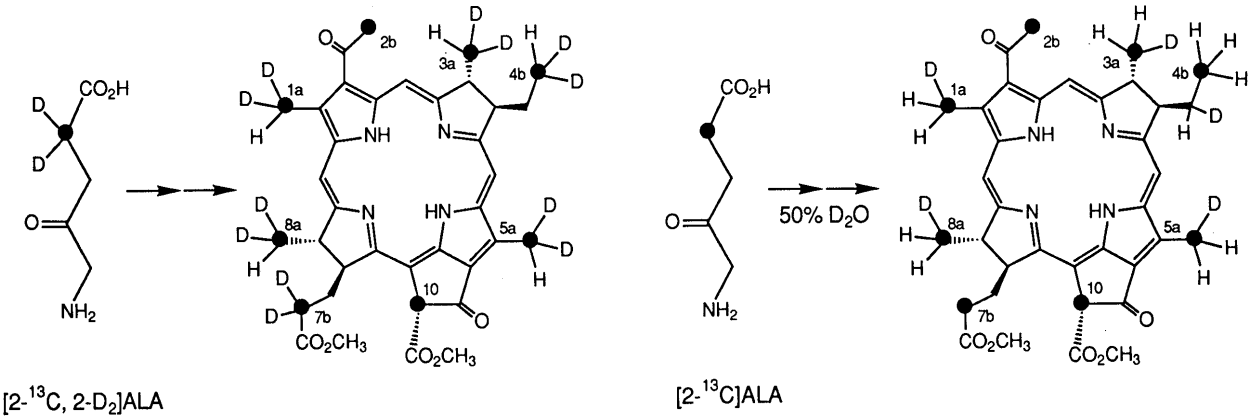

Fig. 6. Labeling Patterns of Methyl Bacteriopheophorbide-a Derived from [2-^{13}C, D_2]ALA and [2-^{13}C]ALA/50% D_2O

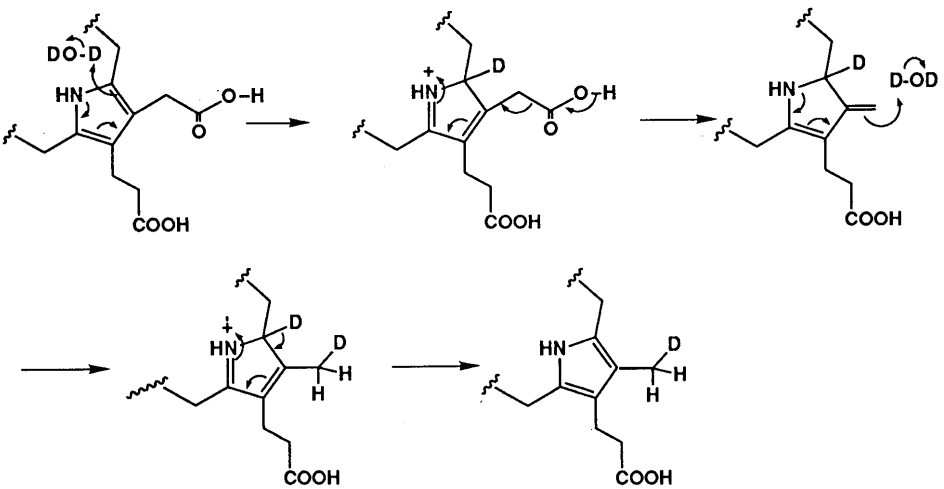

Fig. 7. Hypothetical Decarboxylation Mechanism by Porphyrinogen Carboxy-lyase in 50% D_2O

10 ml) were resuspended in 120 ml (60 × 2) of medium containing a labeled precursor. Amounts of labeled precursor used were as follows: ^{13}C-labeled ALA (60 mg), [2-^{13}C]glycine (60 mg), L-[1-^{13}C] glutamic acid (60 mg), L-[methyl-^{13}C]methionine (50 mg), and ^{15}N-labeled ALA (60 mg). Culture was continued anaerobically in the light for 24—36 h.

Incorporation of ^{13}C-Labeled ALA in Medium Containing 50% D_2O The same procedure as above was used except that the feeding medium contained 50% (v/v) D_2O.

Isolation and Purification of Bacteriochlorophyll-a from Cells Cells were gathered, washed with brine, and disrupted with an ultrasonicator (NIC US-300) at 0 °C for 10 min in methanol solution. The suspension was centrifuged at 10000 rpm at 4 °C for 15 min. The supernatant was evaporated to dryness. The residue was pre-purified by column chromatography (octadecyl silica (ODS), 2.5 i.d. × 25 cm, methanol). The blue-green fraction was collected, evaporated and further purified by HPLC (ODS, 5 μm, 1.5 i.d. × 25 cm, water-methanol) to give 2 mg of pure bacteriochlorophyll-a.

Conversion of Bacteriochlorophyll to Methyl Bacteriopheophorbide Bacteriochlorophyll-a (2 mg) was dissolved in 0.5% sulfuric acid in methanol (10 ml) and left at room temperature for 12 h. Dilution with methylene chloride (200 ml), followed by washing with saturated $NaHCO_3$ solution (2 × 200 ml), water (200 ml) and brine (200 ml), drying (Na_2SO_4), and concentration, gave a residue which was purified by HPLC (silica, 5 μm, 1.5 i.d. × 25 cm, 15 min linear gradient from hexane to isopropanol) to give 1.2 mg of pure methyl bacteriopheophorbide-a.

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