^{13}C-NMR spectra of chlorophylls *a* and *b* in green algae biosynthetically enriched with [1-^{13}C]glutamate

Tamiko Oh-Hama, Haruo Seto, Noboru Otake and Shigetoh Miyachi

Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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5-Aminolevulinic acid *Chlorophyll* a *Chlorophyll* b ^{13}*C-NMR*
[1-^{13}C]glutamic acid Scenedesmus obliquus

1. INTRODUCTION

5-Aminolevulinic acid (ALA), a precursor of the tetrapyrrole compounds, can be formed either by condensation of succinate and glycine which is catalyzed by ALA synthetase, or from the entire carbon skeleton of glutamate (C-5 pathway [1]). In the former, C-5 of ALA is derived from C-2 of glycine, while it is derived from C-1 of glutamate in the C-5 pathway. We reported that ^{13}C of [1-^{13}C]glutamate was preferentially incorporated into the carbon atoms of chlorophyll derived from C-5 of ALA, while that of [2-^{13}C]glycine was into methoxyl group adjacent to the alicyclic ring, in green alga, *Scenedesmus obliquus* in light [2]. However incorporation pattern of ^{13}C in chlorophylls *a* and *b* has not been clarified.

Here, ^{13}C-NMR spectra of chlorophylls *a* and *b* enriched with [^{13}C]glutamate indicated that the incorporation of carbon into four methine bridge carbon atoms (C_α, C_β, C_γ and C_δ, corresponding to C-5, C-10, C-15 and C-20, respectively, according to IUPAC-nomenclature) and four sp_2 quaternary carbon atoms (C-12, C-14, C-16, C-17), derived from C-5 of ALA, was much higher in chlorophyll *a* (fig. 1) than in *b*. Since increase of chlorophyll *b* was almost equivalent to that of *a*, we assumed that chlorophyll *b* was not only synthesized from newly formed chlorophyll *a*, but also from that had been produced before feeding labeled glutamate.

Abbreviation: ALA, 5-aminolevulinic acid

2. MATERIALS AND METHODS

2.1. *Labeling of chlorophylls* a *and* b

Scenedesmus obliquus cells were cultured in the dark for 4 days at 29°C [4]. The algal cells were then suspended in 400 ml phosphate buffer (4 mM,

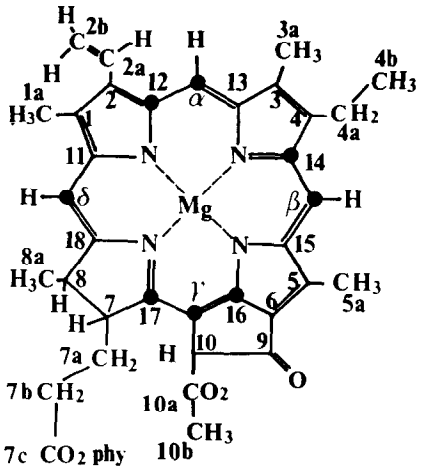

Fig. 1. The structure and numbering system of chlorophyll *a*. Atoms denoted by closed circles (●) are derived from the C-5 of ALA [3].

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pH 6.5) containing 8 mM KNO_3 and 1 mM $MgSO_4$ at 5 ml packed cell vol./l. After 2 h-illumination with daylight fluorescent lamps (10 kLux), [1-^{13}C]glutamate (90 atom%) was added to give 1 mM final conc. The algal cells were illuminated (Xe-lamp, 12 kLux) for 6 h [2], then collected to isolate chlorophylls *a* and *b*. Mixture of chlorophylls *a* and *b* was also isolated after feeding [1-^{13}C] (90 atom%)- and [1-^{14}C]glutamate for 5 h [2].

Chlorophylls *a* and *b* were determined spectrophotometrically [5]. Chlorophyll was isolated as in [2], using DEAE-Sepharose 6B [6]. Chlorophyll *a* was then separated from *b* using Sepharose 6B [6]. Radioactivity in chlorophyll fraction was counted with a windowless gas-flow counter.

2.2. *^{13}C-NMR spectra*

The spectra of chlorophyll in acetone-d_6 were recorded using a JEOL-FX 400 spectrometer under the following conditions: resonance frequency 100 MHz, pulse width 8 μs, repetition time 2.34 s, spectral width 24 000 Hz, 20 500–24 000 transients. Chemical shifts are expressed in ppm relative to TMS. They were derived from the acetone-d_6 methyl-signals as internal standard (29.2 ppm from TMS). Amounts of chlorophyll used for analysis were indicated in each legend.

[1-^{13}C]Glutamate was prepared as in [2]. [1-^{14}C]-Glutamate was from New England Nuclear (Boston MA). DEAE-Sepharose CL-6B and Sepharose 6B were from Pharmacia (Uppsala). Acetone-d_6 was a product of Merck (Darmstadt).

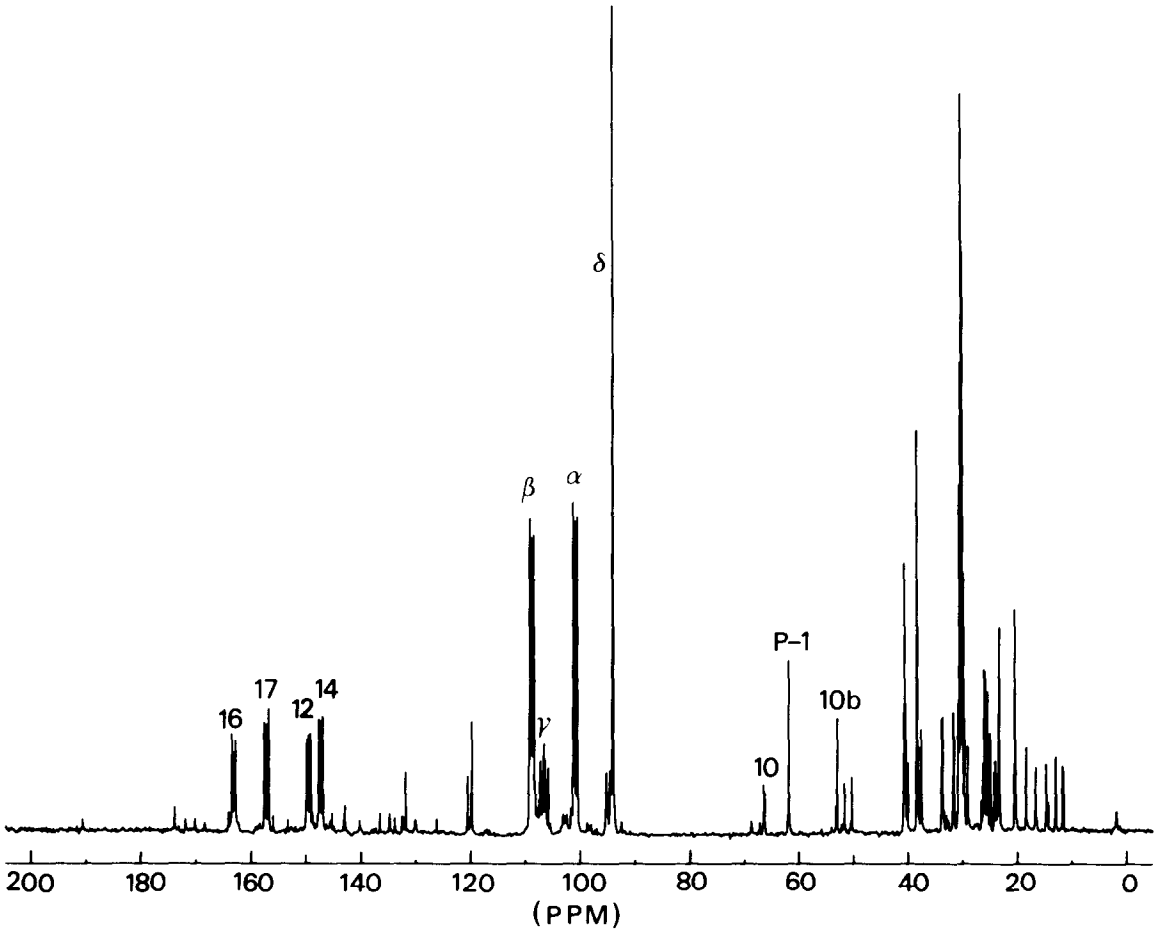

Fig. 2. The ^{13}C-NMR spectrum of chlorophyll *a* in acetone d_6. ^{13}C-Enriched chlorophyll *a* (8.7 mg) was obtained by feeding [1-^{13}C]glutamate to the cells of *Scenedesmus obliquus* for 6 h.

3. RESULTS AND DISCUSSION

When the dark-grown cells of *Scenedesmus* were illuminated with white light (12 kLux), chlorophyll content increased, doubling within several hours [4]. Chlorophylls *a* and *b* were isolated from the algal cells which had been fed [1-^{13}C]glutamate for 6 h, following the pre-illumination which lasted for 2 h. Fig. 2 represents ^{13}C-NMR spectrum of ^{13}C-enriched chlorophyll *a*. The spectrum is similar to that of the total chlorophyll fraction enriched with ^{13}C under similar conditions [2], except that two rather minor peaks in sp_6 methine region (108 and 112 ppm) were eliminated. In [7] assignment of two quaternary carbons, C-16 and C-17, was revised as shown in fig. 2, using a technique of long-range selective ^{1}H decoupling [7]. The present results indicate that ^{13}C of glutamate was specifically incorporated in eight carbon atoms in chlorophyll *a* derived from C-5 of ALA. In [8], ^{13}C of [1-^{13}C]glutamate was incorporated into the chlorophyll macrocycle in a manner consistent with the operation of the C-5 pathway in excised, etiolated maize leaves.

Fig. 3 represents the 4-fold expanded spectra of chlorophylls *a* and *b* in the protonated and quaternary sp_2 carbon regions. Signals from most of the ^{13}C-enriched carbon atoms of both chloro-

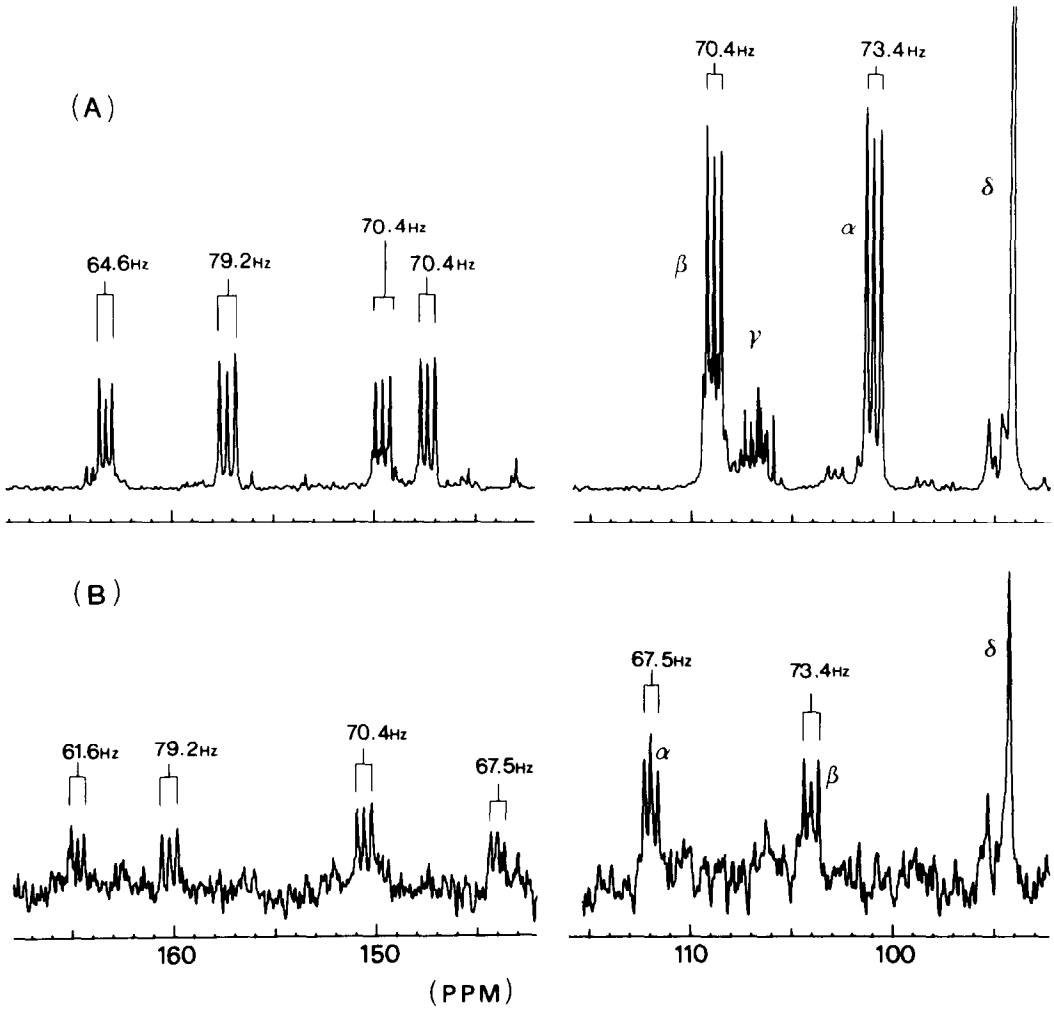

Fig. 3. Expanded spectra of chlorophyll *a* (A) and *b* (B), in the protonated and quaternary sp_2 carbon regions. Chlorophyll *a* (8.7 mg) and chlorophyll *b* (left, 2.4 mg; right, 1.3 mg).

phylls *a* and *b* were split into doublets. The splitting patterns and coupling constants of these carbons are shown in fig. 3. Chemical shifts at 93.0, 99.9, 105.6 and 107.8 ppm for chlorophyll *a* correspond to δ, α, γ and β carbon atoms, and those at 93.1, 102.8 and 110.7 ppm for chlorophyll *b* to δ, β and α carbon atoms, respectively, according to early assignments of ^{1}H-NMR of methylpheophorbide *b* and pheophytin *b* [9] and ^{13}C-NMR of chlorophyll *b* [10]. Doublets of 4 quarternary carbon atoms enriched with ^{13}C in chlorophyll *a* are centered at 146.3 (C-14), 148.5 (C-12), 156.1 (C-17) and 162.1 ppm (C-16); and those of chlorophyll *b* are of 142.9, 149.5, 159.1 and 163.7 ppm, respectively. In both pigments the ratios between the intensities of the doublet and singlet are the same (i.e., ~2:1) indicating that the relative incorporation of ^{13}C into sp_2 carbons in chlorophylls *a* and *b* molecules are the same. Multiple splitting was observed with the γ-methine carbon in chlorophyll *a*, which can arise from interactions with ^{13}C-16 and ^{13}C-17. The multiple splitting in chlorophyll *b* would have caused vague multiplet patterns of the signals as shown in fig. 3B.

Chlorophyll *b* is synthesized from chlorophyll *a* [11]. With *Scenedesmus* cells incorporation of labeled carbon atoms into chlorophylls *a* and *b* were studied in the presence of [1-^{14}C]- and [1-^{13}C]glutamate. Radioactivity in the chlorophyll fraction increased linearly for 5 h from the beginning, and chlorophyll *b* was produced during the second half period of the labeling time. Fig. 4 represents ^{13}C-NMR spectrum of chlorophyll thus labeled after 5 h. It is clear that ^{13}C of glutamate was 5–6-times greater in C_α and C_β of chlorophyll *a* than those of *b*. Chlorophyll *b*, however, comprised 40% of the total chlorophyll produced (table 1). Specific radioactivity in chlorophyll *a* produced during incubation with [1-^{14}C]glutamate was 4-times greater than that in *b*, indicating higher dilution in *b* than in *a*. This indicates that chlorophyll *b* was not only synthesized from newly formed chlorophyll *a*, but also from that having been produced before feeding [^{13}C]glutamate.

Chlorophyll *b* would be finally incorporated into light-harvesting chlorophyll *a*/*b* protein [12]. Shlyk [11] has proposed that biosynthesis of chlorophyll *b* can be synthesized only from a pool of newly synthesized chlorophyll *a* molecules. Consistently with that proposal, it was reported [13] that,

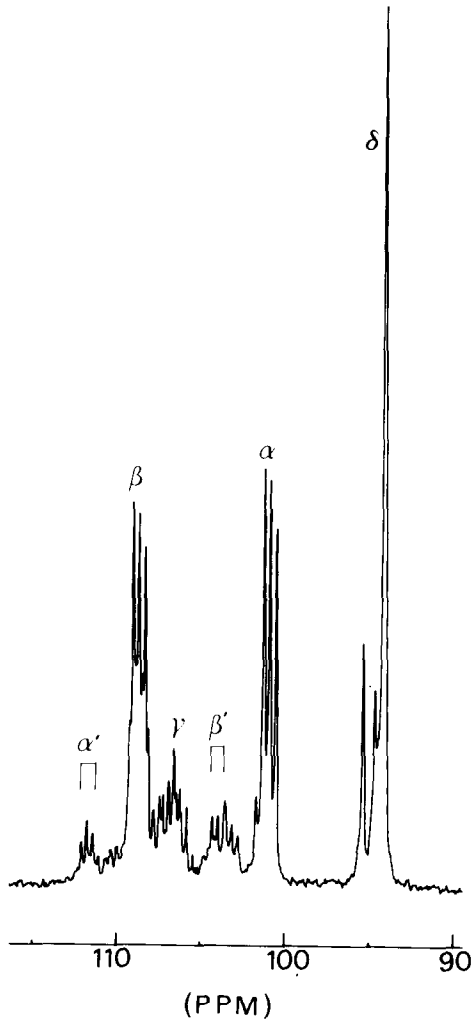

Fig. 4. ^{13}C-NMR spectrum of chlorophyll enriched with ^{13}C by feeding [1-^{13}C]glutamate to algal cells for 5 h: α, β, γ and δ are methine bridge carbons of chlorophyll *a*; α' and β', those of chlorophyll *b*; 8.4 mg chlorophyll was used.

when leaves of *Phaseolus* accumulating [^{14}C]-chlorophyll *a* by intermittent illumination were transferred to continuous light in the absence of [^{14}C]ALA, the leaves produced chlorophyll *b*, in which no ^{14}C existed. In contrast, chlorophyll *a*, pre-accumulated in cucumber seedlings by intermittent illumination, was continuously converted to chlorophyll *b* during the subsequent dark periods provided $CaCl_2$ was present [14]. It is known that chlorophyll *a* formation in green algae proceeds in total darkness, while that in angio-

Table 1

Contents of chlorophylls *a* and *b* in dark-grown cells of *Scenedesmus* and increments of those after exposure of light in the absence, then in the presence of [1-^{13}C]- and [1-^{14}C]glutamate

	Chlorophyll (μmol)	
	a	*b*
Dark-grown cells (μmol/ml cells)	3.67	1.55
Produced during 2 h pre-illumination	0.9	n.d.
Produced during 5 h labeling	1.87	1.39

n.d., not detectable

sperms requires light. Results described above suggest that formation of chlorophyll *b* in angiosperms can be different from that in green algae, but mimic it under a certain condition.

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