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^{13}C-NMR EVIDENCE FOR THE PATHWAY OF CHLOROPHYLL BIOSYNTHESIS IN GREEN ALGAE

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SUMMARY: Analysis of the ^{13}C-NMR spectra of chlorophyll enriched by feeding [1-^{13}C]glutamate or [2-^{13}C]glycine to a green alga, Scenedesmus obliquus, showed that ^{13}C of the glutamate was incorporated in eight carbon atoms in a tetrapyrrole macrocycle derived from C-5 of 5-aminolevulinic acid (ALA), while ^{13}C of glycine was in the methoxyl group adjacent to the isocyclic ring. A reversion was proposed in assignment of ^{13}C chemical shifts of quaternary carbon in chlorophyll a.

INTRODUCTION

ALA, a precursor of 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, Ref. 1). In the former pathway, 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. Both pathways have been assumed to contribute to chlorophyll biosynthesis in green algae (2,3). In these experiments ALA was accumulated by blocking its conversion to porphobilinogen through the addition of levulinic acid, and the location of the label within the ALA molecule was determined after incubating the specifically ^{14}C-labeled precursors. However, we observed that compounds such as glucose and glycine stimulated ALA accumulation in the presence of levulinic acid (4). Correspondingly, Kah et al. (5) reported that levulinic acid stimulated the activity of ALA synthetase. Thus, ALA accumulation in the presence of levulinic acid may not properly reflect chlorophyll formation under natural conditions.

Labeling experiments with ^{13}C offer advantages over ^{14}C for biosynthetic work provided that NMR signals from the carbon atoms of interest can be assigned and that sufficient ^{13}C-labeled compounds are produced for

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spectroscopic study. The chemical shifts of all carbon atoms in chlorophyll a have been assigned (6). Eight C-5 carbons of ALA have been reported to be in positions marked with closed circles in the chlorophyll a molecules shown in Fig. 1 (7). Using [1-^{13}C]glutamate and [2-^{13}C]glycine as tracers and Scenedesmus cells which rapidly synthesize chlorophyll, we studied the biosynthetic pathway of ALA without blocking chlorophyll synthesis. The results indicated that the C-5 pathway is exclusively operating for chlorophyll synthesis in green algae in light.

MATERIALS AND METHODS

Scenedesmus obliquus cells were cultured in the dark for 4 days at 29° C (8). After 2 hr illumination (daylight fluorescent lamps, 10 klux) to induce chlorophyll formation, the algal suspension was centrifuged and suspended in phosphate buffer (4 mM, pH 6.5) containing 8 mM KNO_3 and 1 mM $MgSO_4$ (9). The suspension was divided into three flat glass vessels (the algal suspension, 350 ml each; cell density, 5 ml packed cell volume/liter), and C-1 labeled glutamate or C-2 labeled glycine was added to give a final concentration of 1 mM. Both labeled carbons contained 90 atom % enriched with ^{13}C and 0.14 mCi ^{14}C/mmole C. Cells were allowed to metabolize the added compounds for 5 hr in light (Xe-lamp, 12 klux) under the bubbling of air. The pH of the medium was adjusted to the initial value at 1 hr intervals by adding 0.1 N HCl.

Isolation of chlorophyll: Chlorophyll extracted in methanol was determined as described previously (9). The methanol extracts were mixed with an equal volume of ethyl ether, then sufficient water was added to transfer pigments into the ether phase which was evaporated to dryness under reduced pressure. The pigments were dissolved in acetone and chromatographed on DEAE-Sepharose-6B (10). Radioactivities in carotenoid and chlorophyll fractions were counted with a windowless gas flow counter. The chlorophyll fractions were dried and dissolved in acetone-d_6 for use in ^{13}C-NMR analysis.

N-Chloroacetyl-D,L-[1-^{13}C]glutamic acid (90 atom % ^{13}C), prepared from 90 atom % [^{13}C]KCN (Prochem, London) by a modified method of Mezö et al. (11) was hydrolyzed with hog kidney acylase (Sigma Chem. Co.). L-Glutamic acid was separated by Amberlite IR-120 [H^+]. Specific ^{13}C-enrichment of C-1 of glutamic acid was confirmed by NMR analysis. [2-^{13}C]glycine (90 atom % ^{13}C) was purchased from Merck, Sharp & Dohn, Canada, Ltd., Montreal; [1-^{14}C] glutamic acid and [2-^{14}C]glycine were from New England Nuclear Co., Boston; DEAE-Sepharose CL-6B was from Pharmacia Fin Chem., Uppsala.

RESULTS AND DISCUSSION

The carbon atom numbering system used for chlorophyll a is given in Fig. 1. ^{13}C-NMR spectrum of chlorophyll enriched with [1-^{13}C]glutamate is shown in Fig. 2A. Comparison of the intensities of the enhanced peaks with those at natural abundance (Fig. 2C) indicated a pronounced enrichment in eight carbon atoms. According to early assignment (6), these are four meso-carbon atoms and four quaternary carbon atoms at C-6 (indicated as C-17 in the figure), C-16, C-12 and C-14. The pattern of labeling observed

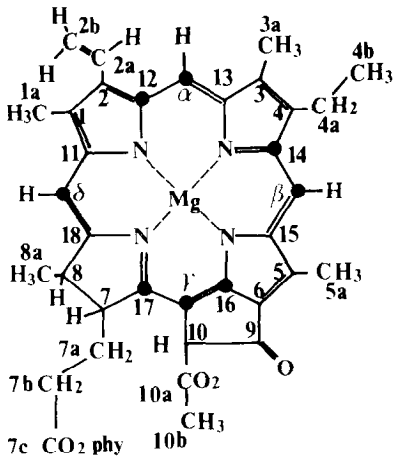

Fig. 1. The structure of and numbering system for chlorophyll a. Atoms denoted by closed circles (•) are derived from the C-5 of ALA (7).

corresponds to that indicated by closed circles in Fig. 1, with the exception of C-6. No enrichment was observed in the position of C-17 (172.2 ppm) which had been assigned by Boxer et al. (6). We assume that the carbon atom at 163.1 ppm is due to C-17 rather than C-6. Boxer et al. (6) have reported that some ambiguities exist in assignment of these C-6, C-16 and C-17 carbon atoms; reinterpretation of the ^{13}C chemical shift of these atoms should be necessary.

Signals from most of the ^{13}C-enriched carbon atoms were split into doublets owing to spin-spin coupling with adjacent ^{13}C atoms. The fine detailed coupling pattern and coupling constants are inserted in Fig. 2A. The ratios between the intensities of the doublets and singlets are about 2:1, indicating that approximately 66% of the ^{13}C-methine carbon atoms (α and γ) and quaternary carbon atoms (C-12, 14, 16 and 17?) were flanked by ^{13}C. This indicates 73% incorporation of C-1 of external added glutamate. Scott et al. (12) observed multiple splitting in γ-meso-carbon of cyanocobalamin in bacterial cells which had been fed [5-^{13}C]ALA. The same multiple splitting was observed in this study.

Fig. 2B shows the ^{13}C-NMR spectrum of chlorophyll enriched with [2-^{13}C] glycine. Conspicuous enrichment of ^{13}C was observed in C-10b, which is known to derive from the methyl-group of S-adenosyl-L-methionine, an inter-

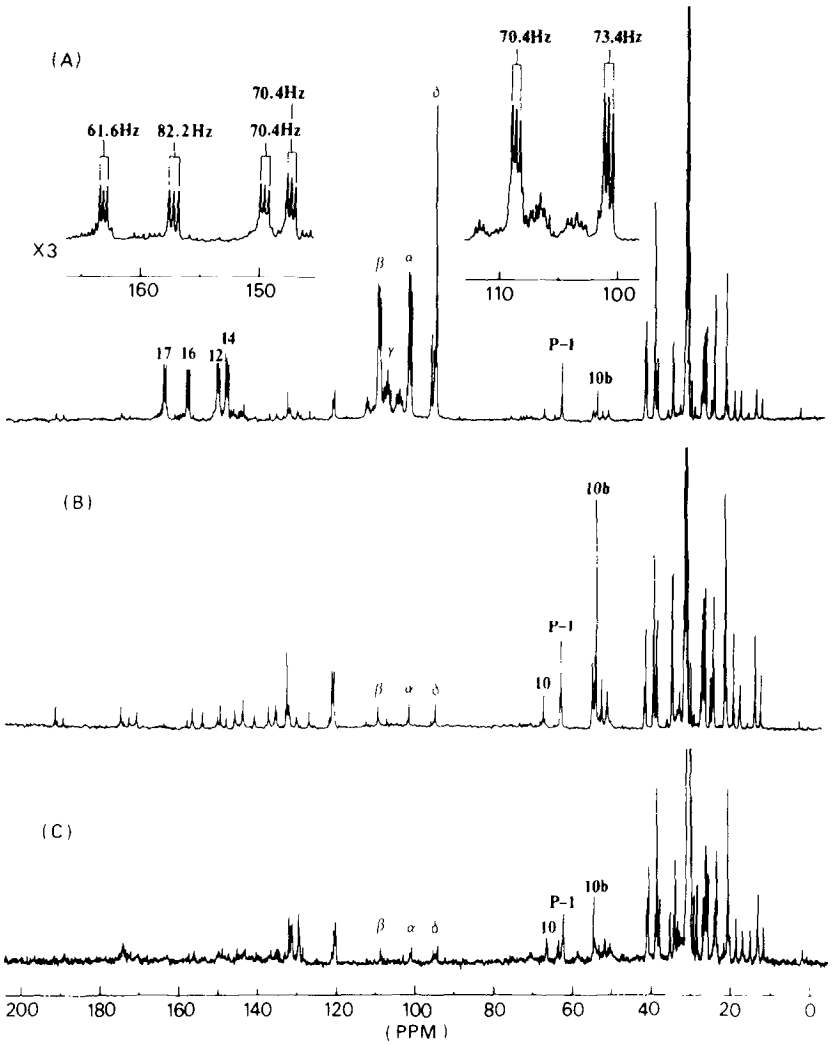

Fig. 2. The ^{13}C-NMR spectrum of chlorophyll in acetone-d_6. Chlorophyll was isolated from Scenedesmus cells after 5 hr-incubation with [1-^{13}C]glutamate (A) or [2-^{13}C]glycine (B) or without feeding a tracer (C) in light. Spectra were obtained at 100 MHz using a JEOL-FX400 spectrometer: pulse width, 8 μsec; 24,000 transients; samples were contained in 5-mm tubes (about 30 mM in acetone-d_6). Chemical shifts are expressed in ppm relative to aceton-d_6 (30.3 ppm from TMS).

mediate in the biosynthesis of magnesium protoporphyrin monomethyl ester. This methyl group transfer reaction is catalyzed by a membrane bound enzyme in the chloroplasts (13).

^{13}C atom of glycine (C-2) may enter into the 10b position as follows:

[2-^{13}C]glycine $\xrightarrow{(I)}$ [3-^{13}C]serine $\xrightarrow{(II)}$ 5, 10 [methylene-^{13}C] methylenetetrahydrofolic acid (FH4) $\xrightarrow{(III)}$ 5 [methyl-^{13}C]methyl-FH_4 –

$\xrightarrow{(IV)}$ [methyl-^{13}C]methionine. The enzyme which catalyzes reaction (I) was found in mitochondria (14) in green plants and also in chloroplasts (15). Enzymes for reactions (II) and (III) exist in chloroplasts (15,16).

From these results we concluded that ALA as a chlorophyll precursor is produced from glutamate via C-5 pathway, but not from succinyl-CoA and glycine in light, and that C-atom at 10b position of chlorophyll is derived from glycine. As mentioned above, an uncertainty exists in the assignments of chemical shifts in C-17 quaternary carbon, but this would not affect this biosynthetic conclusion. Kipe-Nolt and Stevens (17) have observed that the carbon skeleton of ALA in blue-green algae is directly derived from glutamate. However, a possibility that the pathway from glycine and succinyl-CoA is also functioning in the ALA synthesis of this alga cannot be excluded, since the algae cannot take glycine into its cells.

During 5 hr incubation with labeled precursors, 6.3 μmoles of chlorophyll were produced in each sample. From radioactivity incorporated in chlorophyll and neglecting discrimination among ^{12}C, ^{13}C and ^{14}C, we found that the amounts of carbon derived from glutamate and glycine were 6 and 3.6 μmoles per μmole chlorophyll produced, respectively. The latter figure is too high if one assumes that the carbon atom from glycine is incorporated only into 10b. Fig. 2 shows that the location of glycine-^{13}C in aliphatic carbons was much greater than that of glutamate-^{13}C. In the aliphatic region, there were a large number of phytol resonance (18) which overlapped those of the methyl and methylene group in the macrocycle. We found that much more carbon was transferred to the carotenoid fraction from ^{14}C-glycine than from ^{14}C-glutamate (data not shown). Biosyntheses of carotenoid and phytol share a common precursor, mevalonate (19). It is therefore very likely that the ^{13}C spectrum in the aliphatic region was enriched with phytol carbon atoms in a case where glycine was fed. Some random labeling is also seen in both ^{13}C-substrates.

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