# Direct Observation of Enzymatic Transformation of δ-Aminolevulinic Acid to Porphobilinogen and Uroporphyrinogen in a Cell-Free System by Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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**The enzymes which transform δ-aminolevulinic acid into porphobilinogen and uroporphyrinogen were isolated and partially purified for use in a cell-free system. A technique was developed for direct observation of the time course of the reactions in this system by carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectroscopy, using $^{13}$C-labeled aminolevulinic acid as the substrate.**

**Keywords** $^{13}$C-NMR; $^{13}$C-aminolevulinic acid; $^{13}$C-porphobilinogen; $^{13}$C-uroporphyrinogen; aminolevulinic acid dehydratase; cell-free system; enzymatic transformation

Aminolevulinic acid dehydratase (EC 4.2.1.24), the second enzyme of the tetrapyrrole biosynthetic pathway, catalyzes the condensation of two molecules of δ-aminolevulinic acid (ALA) into a monopyrrole, porphobilinogen (PBG). The next enzyme of the pathway, porphobilinogen deaminase (EC 4.3.1.8), catalyzes the tetramerization of PBG to hydroxymethylbilane (HMB). Then HMB is isomerized and cylized by uroporphyrinogen III cosynthase (EC 4.2.1.75), to produce uroporphyrinogen III (URO'gen III), which is an essential precursor of vitamin $B_{12}$, chlorophyll and heme (Fig. 1). To observe these pathways directly, we planned to incubate the enzymes and substrate ($^{13}$C-labeled ALA) in an nulcear magnetic resonance (NMR) tube and to follow the reaction course by $^{13}$C-NMR spectroscopy.

## Materials and Methods

The $^{13}$C-labeled ALAs were synthesized as reported in the previous paper,[1] starting from sodium [$^{13}$C]acetate. Aminolevulinic acid dehydratase (ALA-D) was partly purified from rabbit liver by a modification of the procedure of Anderson and Desnick,[2] except that 10 μM zinc sulfate and 0.1 mM dithiothreitol (DTT) were added during the purification. The specific activity of the preparation was 2.23 units/mg of protein, (one unit of enzymatic activity was defined as the amount forming 1 μmol of PBG/h at 37 °C). ALA-D activity was assayed by the procedure of Kondo *et al.*[3]

A cell-free enzyme system which contained a porphyrin-producing enzyme was prepared from *Rhodopseudomonas spheroides*. Cells (20 g wet weight) were sonicated in 0.05 M potassium phosphate buffer (100 ml), pH 7.6, containing 10 μM zinc sulfate and 0.1 mM DTT. After centrifugation (10000 *g*), solid ammonium sulfate was added to the supernatant to give 60% saturation. The mixture was stirred for 1 h and then centrifuged at 10000 *g*. The precipitate was resuspended in the above buffer and clarified by centrifugation (10000 *g*). The supernatant solution was dialyzed against the above buffer (2 l × 2, 5 h each).

Proton-decoupled $^{13}$C-NMR spectra were measured in phosphate buffer in a 5 mm NMR tube using a JNM GSX-400 spectrometer at 100.4 MHz. The mixture (Table I) was rapidly mixed then incubated in an NMR tube. These spectra were run at 20 °C or 37 °C using a pulse width of 5.5 μs (45° flip angle) and spectral width of 25 kHz. Data were accumulated for 1000 scans/h. An internal lock on $D_2O$ was used and the chemical shifts are given relative to dioxane (67.4 ppm).

High performance liquid chromatography (HPLC) analyses of

Table I. Conditions of the Enzyme Reaction

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|---|---|
| Enzyme solution | 0.3 ml |
| $^{13}$C-Labeled ALA | 250—500 μg |
| $D_2O$, 5% dioxane solution | 0.01 ml |
| $ZnSO_4$ (1 mM) and DTT (100 mM) solution | 0.05 ml |
| 0.5 M phosphate buffer (pH 6.4) | 0.05 ml |
| Temperature | 20 °C or 37 °C |

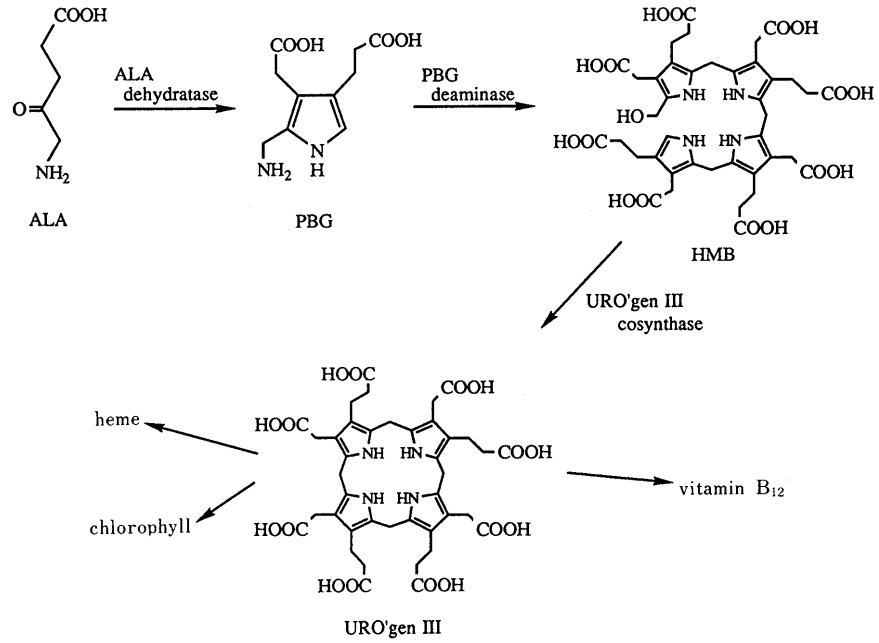


Fig. 1. Biosynthetic Pathway of Heme, Chlorophyll and Vitamin $B_{12}$

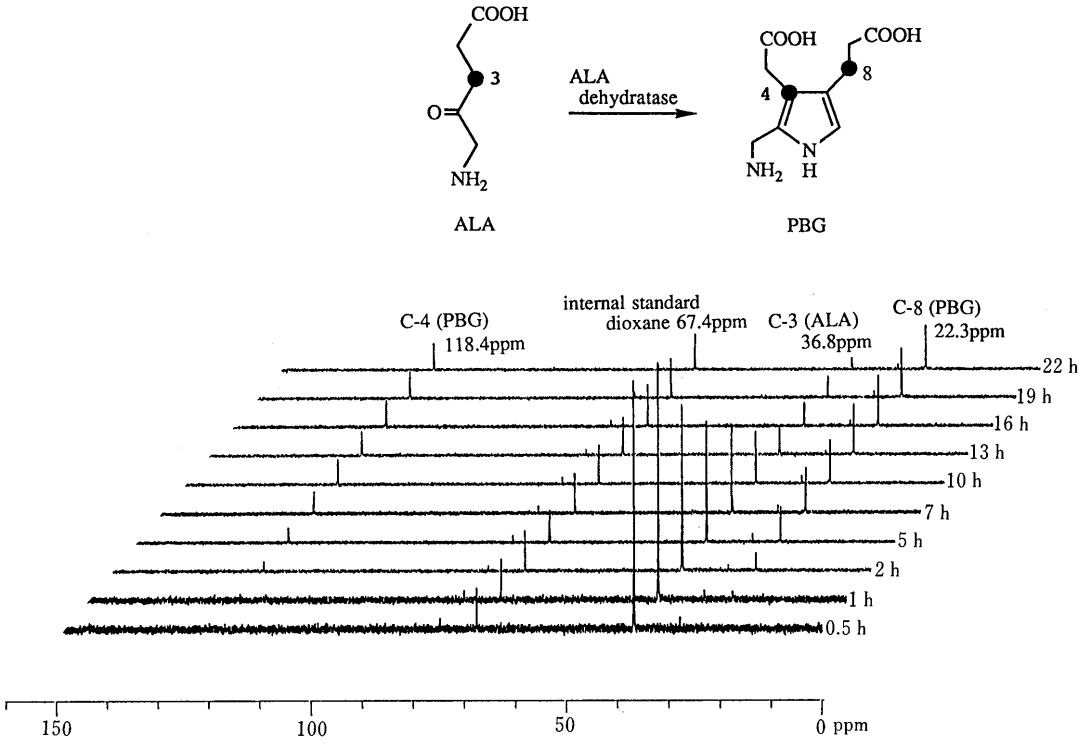


Fig. 2. $^{13}$C-NMR Spectra Illustrating the Time Course of Enzymatic Transformation from ALA to PBG

The conditions are given in Table I ([3-$^{13}$C]ALA, 400 $\mu$g; temperature, 20 °C).

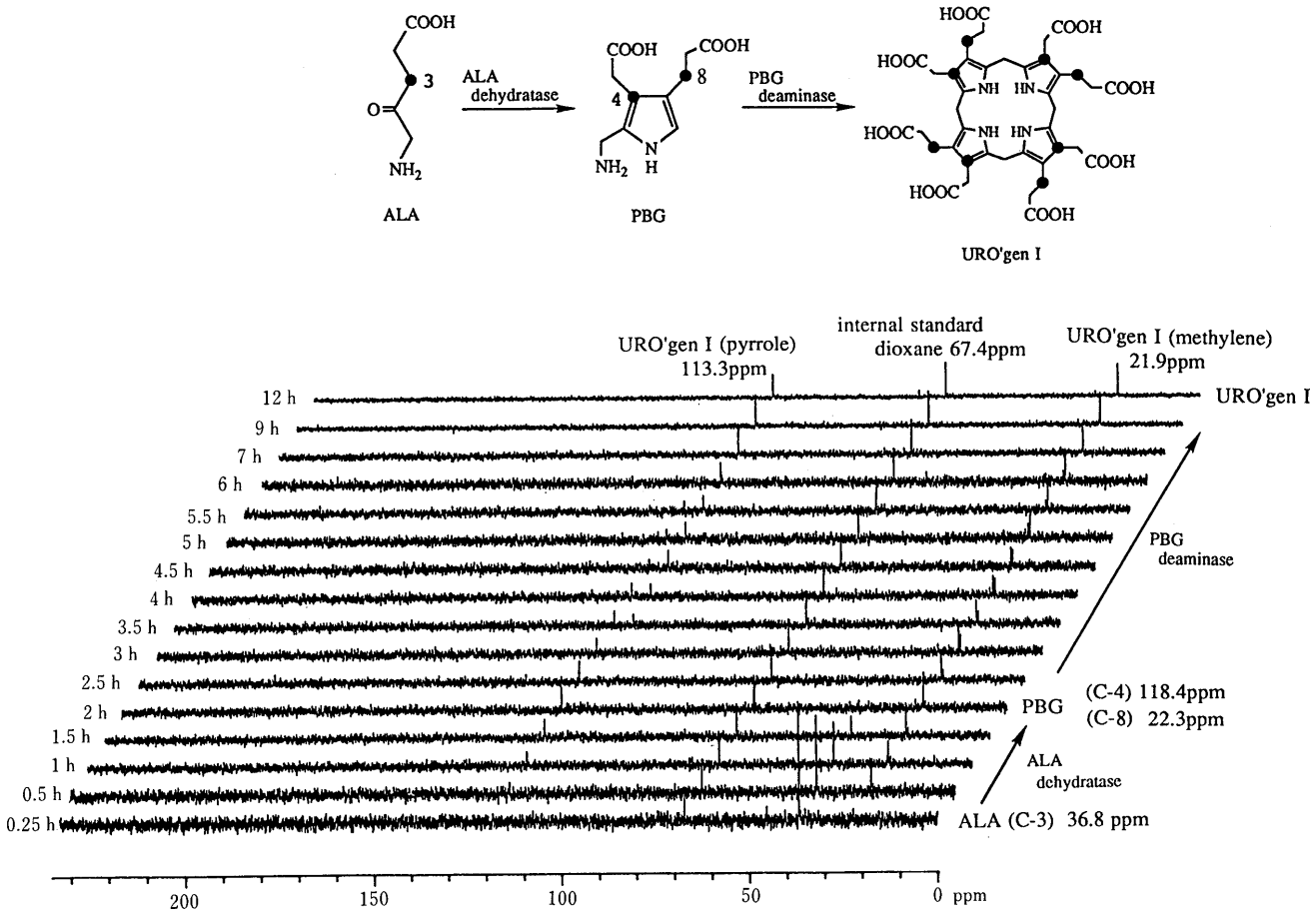


Fig. 3. $^{13}$C-NMR Spectra Illustrating the Time Course of Enzymatic Transformation from ALA to PBG and thence to URO'gen I

The conditions are given in Table I ([3-$^{13}$C]ALA, 250 $\mu$g; temperature, 37 °C).

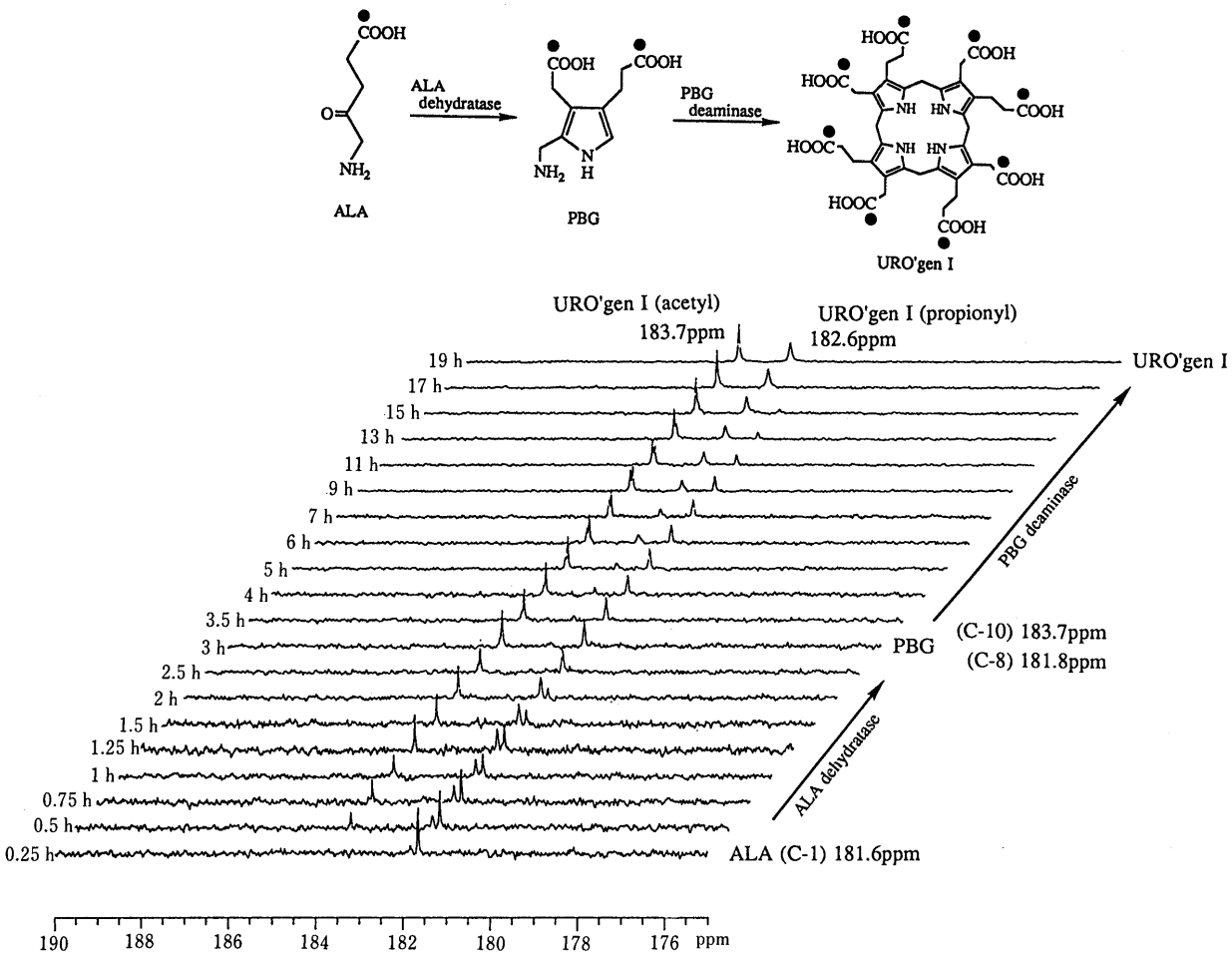


Fig. 4. Expanded $^{13}$C-NMR Spectra Illustrating the Time Course of the Transformation from [1-$^{13}$C]ALA to URO'gen I

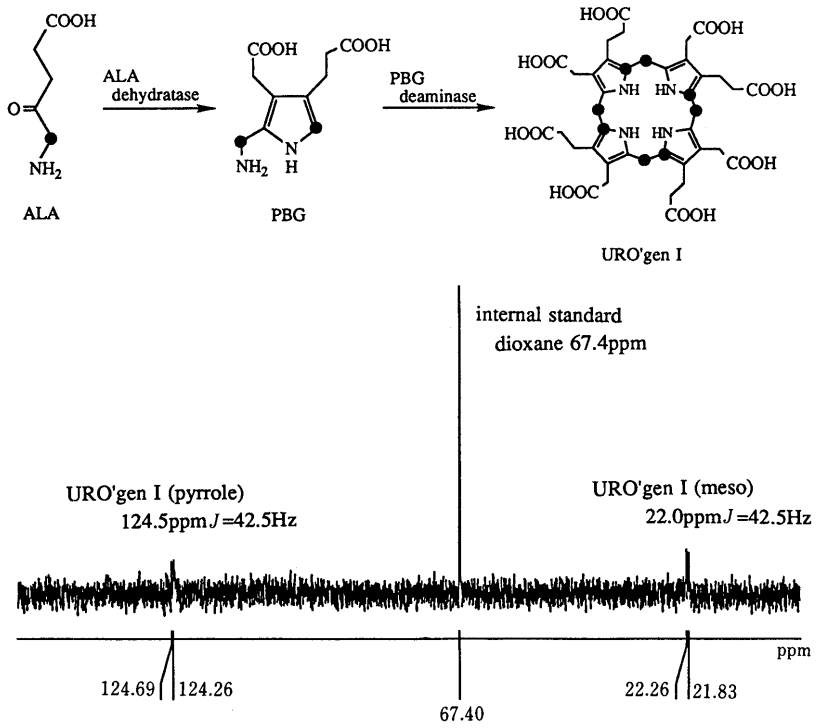


Fig. 5. $^{13}$C-NMR Spectrum Showing Formation of URO'gen I from [5-$^{13}$C]ALA after 12 h
10000 scans.

porphyrins were performed on a 4.6 mm i.d. × 250 mm column packed with Finepak SIL $C_{18}$ or Capcell pak $C_{18}$ (5 $\mu$m) at 25 °C. The mobile phase for the gradient elution was composed of acetonitrile, acetic acid and ammonium acetate. A fluorescence detector was used for detection. The excitation and emission wavelengths were set at 404 and 620 nm, respectively. The flow rate was maintained at 1 ml/min throughout.

## Results and Discussion

Incubation of [3-$^{13}$C]ALA with ALA-D from rabbit liver gave $^{13}$C-labeled PBG. In the $^{13}$C-NMR spectra, the signal of [3-$^{13}$C]ALA at 36.8 ppm disappeared rapidly. On the other hand, the signals at 22.3 and 118.4 ppm, due to [4,8-$^{13}$C]PBG, became larger (Fig. 2).

The $^{13}$C-NMR spectra of the cell-free enzyme reaction are shown in Fig. 3. The signal of [3-$^{13}$C]ALA at 36.8 ppm disappeared at 2 h, while two signals appeared at 22.3 and 118.4 ppm, corresponding to [4,8-$^{13}$C]PBG. These two signals of PBG were replaced completely by corresponding signals of URO'gen I (21.9, 113.3 ppm) 7 h later.

Incubation of [1-$^{13}$C]ALA and [5-$^{13}$C]ALA with the cell-free enzyme system gave similar results (Fig. 4). The $^{13}$C-NMR spectra in the [5-$^{13}$C]ALA experiment showed $^{13}$C–$^{13}$C coupling ($J$=42.5 Hz) corresponding to symmetrical type I URO'gen (Fig. 5). It appears that PBG was transformed to HMB by PBG-deaminase and HMB was then cyclized chemically under the conditions used to give mainly URO'gen I.

Furthermore, HPLC analysis[4] of the reaction mixture (Fig. 6), revealed the presence of other porphyrins such as 8-, 7-, 6-, 5- and 4-carboxylated porphyrins, accompanied with traces of type III porphyrins.

## Conclusion

We have developed a means to follow the time course of enzyme reactions in a cell-free system by using $^{13}$C-NMR spectroscopy. The syntheses of porphobilinogen and uroporphyrinogen from $^{13}$C-labeled aminolevulinic acid in a cell-free system were easily and directly followed. This approach should be useful for studies on the mechanisms of enzyme reactions.

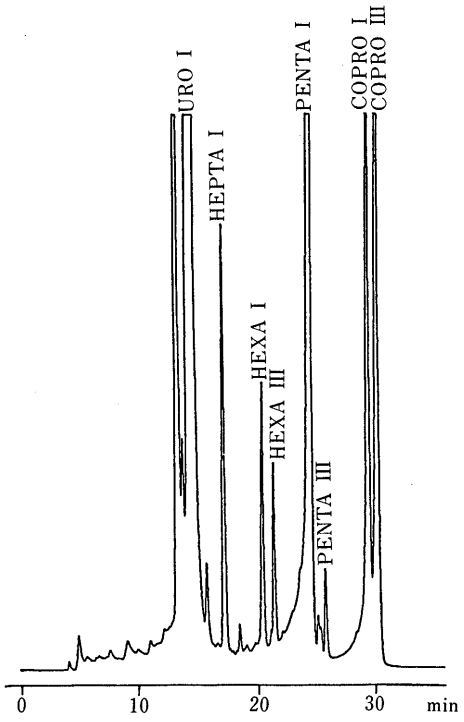


Fig. 6. HPLC Analysis of Cell-Free Enzyme Reaction Mixture

The solvents for the gradient elution were acetonitrile, acetic acid and ammonium acetate. Solution A: 80% acetonitrile/7% acetic acid/50 mM ammonium acetate. Solution B: 10% acetonitrile/4% acetic acid/50 mM ammonium acetate. A 30 min linear gradient from A/B (20/80) to A/B (90/10) was used.

Abbreviations used: URO, 8-carboxylated porphyrin; HEPTA, 7-carboxylated porphyrin; HEXA, 6-carboxylated porphyrin; PENTA, 5-carboxylated porphyrin; COPRO, 4-carboxylated porphyrin.

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