Studies on the Biosynthesis of Corrinoids and Porphyrinoids. III. The Origin of Amide Nitrogen of Vitamin B_{12}

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To clarify the origin of amide-nitrogen of vitamin B_{12}, [1-^{13}C]aminolevulinic acid (ALA) and L-[amide-^{15}N]glutamine were administered to *P. shermanii*. The ^{13}C-nuclear magnetic resonance spectrum of the vitamin B_{12} subsequently isolated showed distinct ^{13}C–^{15}N coupling and isotope shift at six amide carbons. However, the C-57 amide carbon showed neither coupling, nor shift. Thus, it was concluded that the nitrogens of 6 amides of the side chain were derived from glutamine and the C-57 amide nitrogen was from threonine.

Keywords vitamin B_{12}; biosynthesis; nitrogen origin; L-[amide-^{15}N]glutamine; [1-^{13}C]ALA

We have reported on the origin of nitrogen of vitamin B_{12} in the previous paper.[1] In that work, the origins of pyrrole, benzimidazole, and cyano nitrogens could be resolved by the incorporation of ^{15}N-labeled aminolevulinic acid (ALA), riboflavin, or potassium cyanide. They were determined by measurement of the ^{15}N-nuclear magnetic resonance (^{15}N-NMR) spectra of the respective labeled vitamin B_{12}. Only amide nitrogens remained unresolved. In 1986, Eliseev and co-workers presented a report on the source of amide groups in vitamin B_{12} biosynthesis.[2] They administered ^{15}N-labeled glutamine and cobyrinic acid to resting cells of *Propionibacterium shermanii* and isolated the cobinamide. The ^{15}N content in cobinamide was not high (=18%) compared with the theoretical value (=46%). The distribution of ^{15}N was not distinct, so the origin of the amide nitrogen attached to C-57 remained ambiguous (from glutamine or from threonine[3]). Furthermore, we reported previously that ^{18}O of the amide at C-27 had been lost in the biosynthesis of vitamin B_{12} from ^{18}O-labeled ALA.[4] We wanted to investigate the timing of this loss of ^{18}O, *i.e.*, to establish whether it occurred before amidation or not. So we intended to isolate the ^{15}N-labeled vitamin B_{12} without degradation and to specify the labeled positions and their label contents. As ^{15}N-NMR measurement seemed to be difficult[5] when ^{15}N-content was low, we chose to incorporate ^{15}N-glutamine (amide-labeled) accompanied with [1-^{13}C]ALA to *P. shermanii* suspension cells.

Results and Discussion

The feeding conditions followed those of our previous experiment on the incorporation of labeled ALA,[1] except that the concentration of buffer was decreased in order to prevent the decomposition of glutamine.[6] Also, the pH was not adjusted, nor was supplementary glucose added to the cells since we wished to keep the apparatus sealed. The isolated vitamin B_{12} was examined by ^{13}C-NMR. The

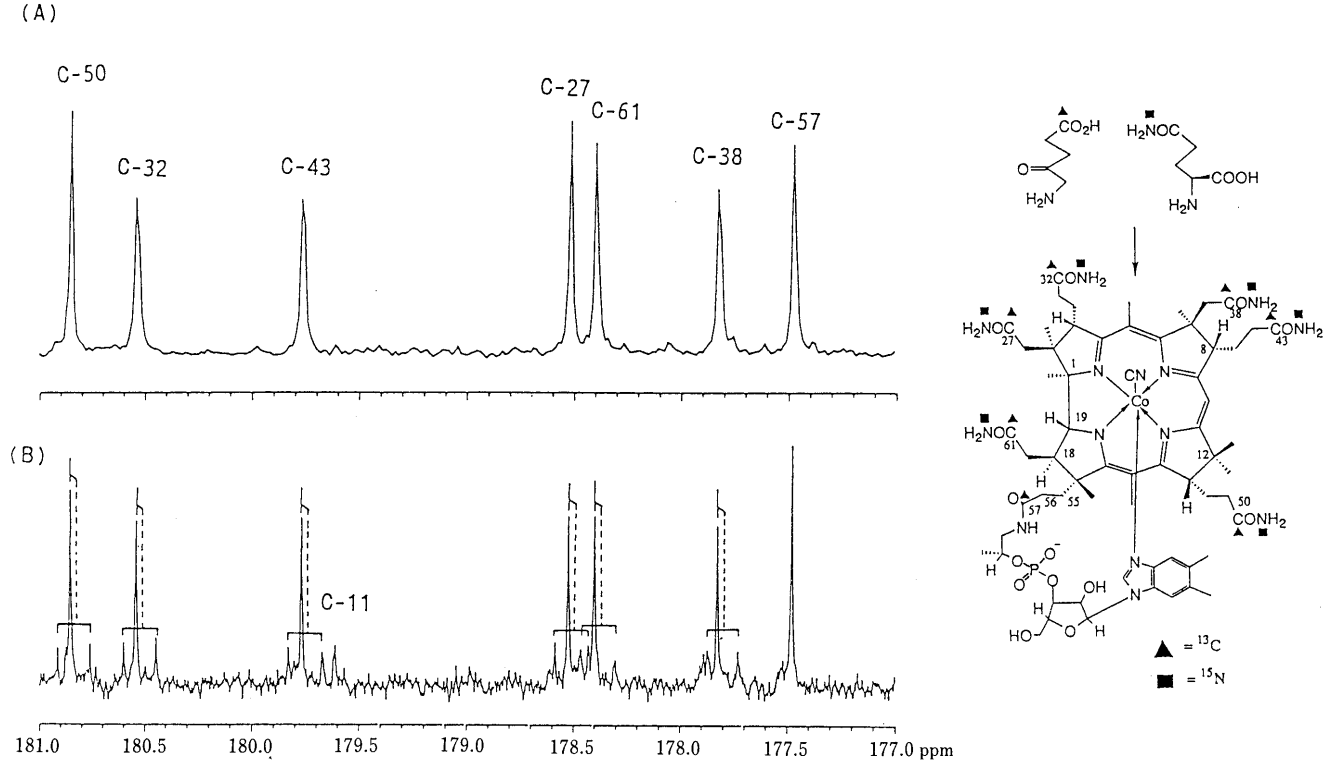

Fig. 1. Comparison of the ^{13}C-NMR Spectrum of [1-^{13}C]ALA-Incorporated Vitamin B_{12} (A) with That of [1-^{13}C]ALA and L-[Amide-^{15}N]glutamine-Incorporated Vitamin B_{12} (B)

spectrum is shown in Fig. 1(B) (bottom). For comparison, the spectrum of the vitamin B_{12} isolated after incorporation of [1-^{13}C]ALA[4] is shown in Fig. 1(A) (top). Six amide carbons (C-50, C-32, C-43, C-27, C-61, and C-38) showed distinct isotope-shifted and split (coupling with ^{15}N) signals. The isotope shifts are 0.016—0.018 ppm (1.6—1.8 Hz), and the coupling constants ($=J_{13C\text{-}15N}$) are in the range of 15.6—16.3 Hz at the corrin ring side chain. From the peak intensity of the original signal and that of the shifted one, the ^{15}N content was estimated. The values of the ratio of peak area of ^{15}NCO/(^{15}NCO+NCO) were 20% (C-50), 29% (C-32), 29% (C-43), 29% (C-27), 27% (C-61), 27% (C-38), 30% (C-57). These values are similar to the result of Eliseev *et al.* (28% = 18/18+46).[2] As the amidation proceeds in a stepwise manner,[7] the low ratio of C-50 might be explained by assuming that the amidation of C-50 was the last of the 6 amidation steps. However, the signal of C-57 showed neither isotope shift nor coupling. This result proves that this nitrogen was derived not from glutamine but from threonine.

Experimental

^{13}C-NMR spectra were taken on a JEOL GSX-400 spectrometer (100 MHz). Chemical shifts are given downfield from sodium [2,2,3,3,-$^{2}H_4$]-3-(trimethylsilyl)propionate (TSP) as an internal standard for ^{13}C-NMR. Ultraviolet (UV) spectra were recorded on a Jasco UVIDEC 610C spectrometer.

Incorporation of L-[Amide-^{15}N]glutamine (2) into Vitamin B_{12}(3) *Propionibacterium shermanii* ATCC 9614 was incubated for 7 d in 12 l of casein I-B medium under a nitrogen atmosphere, with adjustment of the pH to 7.0 every day, and collected by centrifugation at 12000 *g* at 4 °C for 35 min. The cells were washed with brine, and divided into 6 batches (the total weight of wet cells was 210 g), each of which was placed in a 500 ml sterilized flask containing a suitable medium.[8] They were incubated at room temperature for 68 h under the same conditions. The cells were gathered, washed with brine, and disrupted with an ultrasonicator (NIC US-300) at 0 °C for 15 min in 400 ml of 80% methanol solution containing 0.1% potassium cyanide, twice. The suspension was centrifuged at 12000 *g* at 4 °C for 30 min. The supernatant was concentrated to 150 ml, then extracted with 1 : 1 phenol–chloroform (40 ml × 2). The extract was washed with water (50 ml × 2), diluted with 1000 ml of ether, and re-extracted with water (50 ml × 3). The extract was washed with 50 ml of chloroform, then with 50 ml of ether, then evaporated. The residue was purified by column chromatography (SiO_2, methanol) (11 g, 1.5 cm i.d. × 13 cm), and the red fraction (Rf=0.2) was collected and evaporated. The residue was recrystallized repeatedly from water–acetone (1 : 8) to give 2.2 mg of **3** as needles. UV λ_{max} nm: 550.0, 358.4. ^{13}C-NMR (100 MHz, D_2O, enriched peak, TSP) δ: 180.85 (s, C-50), 180.83 (d, ^{15}NC-50, $J_{15N\text{-}C}$ = 15.6 Hz, 0.018 ppm shifted), 180.54 (s, C-32), 180.52 (d, ^{15}NC-32, $J_{15N\text{-}C}$ = 15.8 Hz, 0.018 ppm shifted), 179.77 (s, C-43), 179.75 (d, ^{15}NC-43, $J_{15N\text{-}C}$ = 15.9 Hz, 0.017 ppm shifted), 179.61 (s, C-11, natural abundance peak), 178.52 (s, C-27), 178.51 (d, ^{15}NC-27, 0.016 ppm shifted), 178.40 (s, C-61), 178.37 (d, ^{15}NC-61, 0.016 ppm shifted), 177.83 (s, C-38), 177.81 (d, ^{15}NC-38, 0.017 ppm shifted), 177.48 (s, C-57).

References and Notes

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5) Measurement of the ^{15}N-NMR spectrum of 2—3 mg of pyrrole–nitrogen or benzimidazole–nitrogen (85—90% enriched) took 32—36 h. See ref. 1.
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8) Each flask contained 100 ml of 1.15 mM phosphate buffer (pH = 7.0), 1 mg of CoCl, 3.2 ml of 50% glucose, 30 mg of methionine, 15 mg of [1-^{13}C]ALA (prepared as described in ref. 9), 15 mg of L-[amide-^{15}N]glutamine (obtained from Shoko Tusho Co., 95 atom% ^{15}N) 25 mg of dimethyl benzimidazole.
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