SYNTHESIS OF L- [^{15}N]ALANINE BY THE USE OF A LINKED ENZYME SYSTEM

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1. Introduction

We are interested in the use of ^{15}N-labelled L-amino acids for the study of amino acid metabolism and are therefore investigating biological methods by which these labelled amino acids may be produced.

The synthesis, in gram quantities, of L-[^{15}N] aminobutyric acid, L-[^{15}N]glutamic acid, L-[^{15}N] methionine and L-[^{15}N]valine, by means of beef liver glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (G6PD) in a linked enzyme system have already been reported [1,2]. We here report the synthesis of L-[^{15}N]alanine using GDH and G6PD further linked to glutamic-pyruvic transaminase (fig.1.).

2. Materials and methods

The reaction mixture contained, in 600 ml 0.1 M phosphate buffer, pH 7.6, 26 mmol glucose-6-phosphate (BDH, Na-salt), 6 mmol α-ketoglutaric acid (Sigma, Na-salt), 36 mmol pyruvic acid (Sigma, Na-salt), 24 mmol [^{15}N]ammonium chloride (95 atom% ^{15}N, Prochem BOC) and 30 μmol NADP (Sigma, Na-salt). The pH of the mixture was then adjusted to 7.6 with 2 M KOH and the following enzymes were added: 300 units (IU) glucose-6-phosphate dehydrogenase (Sigma type 15, baker's yeast, lyophilized), 900 units (IU) glutamate dehydrogenase (Sigma, bovine liver, type 2, free from ammonium ions) and 300 units (IU) glutamic-pyruvic transaminase (Sigma, lyophilized). The reaction was run for 16 h at 35°C in a sealed vessel. During the course of the reaction the pH fell from 7.6 to 6.8.

At the end of 16 h the reaction mix was heated to 80°C for 10 min to inactivate the enzymes present. The mix was then cooled to 37°C and the pH adjusted to 5.0 by the addition of 5 M HCl. Glutamic decarboxylase (BDH), 130 IU, was then added and the mix was stirred for 6 h with a N_2 flow flushing the atmosphere of the reaction vessel to remove CO_2. This treatment converted glutamic acid (formed from α-ketoglutarate) to 4-aminobutyric acid which can be easily separated from alanine.

The mix was again heated to 80°C for 10 min, cooled, filtered and applied to a (45 × 3 cm) cation-exchange column (Amberlite IR 120 H^+). The adsorbed amino acids were eluted with 0.2 M KOH using the ninhydrin reaction to follow the elution pattern. The

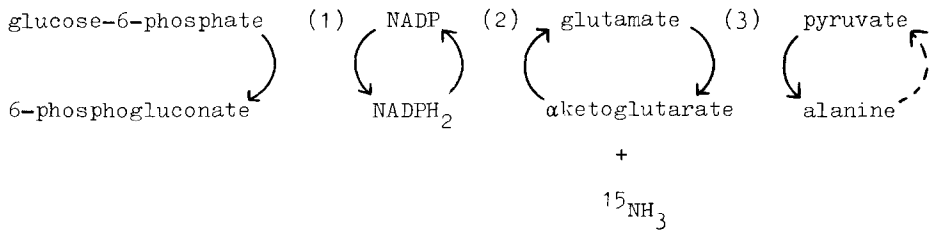

Fig.1. Pathway of synthesis of L-[^{15}N]alanine using a linked enzyme system. (1) Glucose-6-phosphate dehydrogenase; (2) Glutamate dehydrogenase; (3) Glutamic-pyruvic transaminase.

eluted fractions containing amino acids were applied to a (45 × 3 cm) anion exchange column (Amberlite IRA 400 OH^-) and the adsorbed amino acids eluted with 0.2 M acetic acid. The fractions of the eluate containing amino acids were concentrated in a rotary vacuum evaporator at 50°C to dryness. The residue was dissolved in 10 ml distilled water, 90 ml absolute ethanol was added and the mixture was kept at 5°C for 48 h while L-[^{15}N]alanine crystallized. Under these conditions 4-aminobutyric acid did not crystallize. The crystals were filtered off, rinsed with ethanol and dried in vacuo.

The filtrate was dried down in vacuo to recover the 4-[^{15}N]aminobutyric acid together with a small amount of uncrystallized L-[^{15}N]alanine.

2.1. *Analytical techniques*

Gas chromatographic analysis was performed with a Pye 104 gas chromatograph with FID using a 1.5 m × 2 mm i.d. column containing 5% OV1 on 100–120 mesh Gas chrom Q. Amino acids were chromatographed as the heptafluorobutyryl isobutyl ester derivatives.

Optical rotations were measured with a Perkin-Elmer 141 polarimeter using a 10 cm light path.

A VG Micromass 16F mass spectrometer interfaced via a glassjet separator to a Pye 104 gas-chromatograph was used to confirm isotopic composition of the amino acid heptafluorobutyryl isobutyl ester derivatives.

3. Results

The yield of L-[^{15}N]alanine was 1.28 g. $[\alpha]_D^{20°C}$ + 13.5 (c = 1 in M HCl).

Found:

C 40.18; H 7.82; N 16.82.

Calculated for $C_3H_7NO_2$ (95 atom% ^{15}N):

C 40.02; H 7.84; N 16.60.

Mass spectrum *m/e* 240 (5), 241 (95). By gas-liquid chromatography (GLC) > 99% pure.

Concentration of the filtrate from which L-[^{15}N] alanine crystals had been filtered yielded a further 500 mg of amino acids. By GLC analysis this mix contained 90% 4-aminobutyric acid and 10% alanine. A total of 19 mmol $^{15}NH_3$ (79% of theory) was therefore incorporated into amino acids during the reaction from which 14.2 mmol (59% of theory) of pure L-[^{15}N]alanine was crystallized.

4. Discussion

A linked enzyme system which produced μmol amounts of L-alanine has been previously described by Kitai et al. [3]. In their system the reaction was 'driven' (i.e., reduced nucleotide was regenerated) by the conversion of ethanol to aldehyde by alcohol dehydrogenase. We prefer the system described in this paper because the reaction catalysed by G6PD is irreversible (a 'one way drive') whereas that catalysed by alcohol dehydrogenase is reversible.

We have varied the concentrations of reactants in the mix to try to further increase the yield of L-[^{15}N] alanine but without success. We find that the comparatively large amount of α-ketoglutarate added is necessary for the reaction to proceed.

We have had difficulty in completely separating L-glutamic acid from L-alanine. This separation is essential as both amino acids are present at the termination of the reaction. Weak anion exchange resins, such as Amberlite IR-45 (OH^-), which retain dicarboxylic acids such as glutamate acid but not monocarboxylic acids such as alanine, have not yielded complete separations for us. Of those methods which we have tested the conversion of glutamate to 4-aminobutyrate followed by fractional crystallization of alanine from 4-aminobutyrate has provided the most complete separation.

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