SYNTHESIS OF L-[α-^{15}N]AMINOBUTYRIC ACID, L-[^{15}N]METHIONINE AND L-[^{15}N]VALINE BY THE USE OF BEEF HEART GLUTAMATE DEHYDROGENASE

W. GREENAWAY and F. R. WHATLEY
Botany School, South Parks Road, Oxford OX1 3RA, England

Received 10 January 1977

1. Introduction

L-Amino acids labelled with ^{15}N are useful for biological studies of amino acid metabolism [1] but unfortunately they are frequently either commercially unavailable or prohibitively expensive.

Chemical synthesis of the amino acids usually produces a racemic product, although there is occasionally a strong bias towards a single isomer [2]. Enzymic synthesis however can be made to produce the L-isomer alone. Such enzymic synthesis has been carried out by conventional fermentation techniques [3,4] or by biosynthesis using either isolated cells [5,6] or enzyme extracts [7–10]. Unfortunately these methods invariably use materials (e.g., bacterial auxotrophs, dried bacterial cells, tissue extracts) which are not freely available and their application may therefore be limited.

We prefer to attempt enzymic syntheses using only commercially available materials and have recently described such a synthesis of L-[^{15}N]glutamic acid in good yield using beef liver glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase in a linked enzyme system [11].

Beef liver GDH is relatively specific for L-glutamate [12–14] unlike frog heart GDH which can utilize a number of L-amino acids as substrates [13,15]. However the frog heart enzyme is not commercially available. We have therefore attempted the direct synthesis, in gram quantities, of three ^{15}N-labelled amino acids, L-[α-^{15}N]aminobutyric acid, L-[^{15}N]methionine and L-[^{15}N]valine, from their corresponding α-keto acids using beef liver GDH in a linked enzyme system similar to that previously described [11]. These three amino acids were chosen because beef liver GDH has already been shown to utilize them as oxidative substrates, though at low rates [12,13] and because the relevant α-keto acids are freely available commercially.

2. Materials and methods

Reaction mixtures contained, in 400 ml, 0.1 M borate buffer, pH 9.0 [16], 20 mmol glucose-6-phosphate (BDH, Na-salt), 20 mmol α-ketobutyric acid (Sigma, Na-salt), or 20 mmol α-ketomethiolbutyric acid (Sigma, Na-salt), or 20 mmol α-ketoisovaleric acid (Sigma, Na-salt), 20 μmol NADP (Sigma, Na-salt), 20 mmol [^{15}N]ammonium chloride (95 atom% ^{15}N, Prochem BOC), 250 enzyme units (IU) of glucose-6-phosphate dehydrogenase (Sigma type 15, baker's yeast, lyophilized) and 6000 enzyme units (IU) of glutamate dehydrogenase (Sigma, bovine liver, type 2, free from ammonium ions). Reactions were run for 36 h at 30°C in a sealed vessel. The progress of the reaction (in a 500 ml double-neck flask) was monitored by means of a pH-probe sealed into the reaction vessel. When the pH of the reaction mix fell below pH 8.5, 1 M KOH was added by syringe via a rubber septum in order to return the pH to 9.0.

At the end of 36 h the reaction mix was boiled, filtered and applied to a (45 × 3 cm) cation-exchange column (Amberlite IR 120 H^+). The adsorbed amino acid was eluted with [^{14}N]ammonium hydroxide. The fractions of the eluate containing amino acid were concentrated in a rotary vacuum evaporator at 50°C to a small volume (approx. 10 ml). Ninety ml 50/50, v/v, anhydrous ethanol/ether were added and the mixture was kept at −20°C for 2 days, while the

amino acid crystallized. The crystals were filtered off, rinsed with ethanol/ether and dried in vacuo.

2.1. *Analytical techniques*

Gas chromatographic analysis was performed with a Pye 104 gas chromatograph with FID using a 1.5 m X 2 mm ID column containing 5% OV1 on 100–120 mesh Gas Chrom Q. Amino acids were chromatographed as trimethylsilyl and as *N*-dimethylaminomethylene methyl ester derivatives.

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

A VG micromass 12B mass spectrometer interfaced via a glass-jet separator to a Varian gas chromatograph was used to confirm isotopic composition of the amino acid *N*-dimethylaminomethylene methyl ester derivatives.

3. Results

The yield of L-α-aminobutyric acid was 1.41 g (13.7 mmol = 68% of theory). $[\alpha]_D^{20°C} = +18.3$ ($c = 1$ in 5 M HCl). Found: C 46.23, H 8.57, N 14.51. Calculated for $C_4H_9NO_2$ (95 atom% ^{15}N): C 46.17, H 8.72, N 14.37. Mass spectrum *m/e* 113 (6), 114 (94). By gas–liquid chromatography > 99% pure.

The yield of L-methionine was 1.24 g (8.3 mmol = 41% of theory). $[\alpha]_D^{20°C} = +23.1$ ($c = 1$ in 5 M HCl]. Found: C 40.02, H 7.21, N 9.93, S 21.02. Calculated for $C_5H_{11}NSO_2$ (95 atom% ^{15}N): C 40.00, H 7.38, N 9.96, S 21.35. Mass spectrum *m/e* 157 (4), 158 (96). By gas–liquid chromatography > 99% pure.

The yield of L-valine was 0.98 g (8.4 mmol = 42% of theory). $[\alpha]_D^{20°C} = +26.5$ ($c = 1$ in 5 M HCl). Found: C 50.97, H 9.21, N 12.75. Calculated for $C_5H_{11}NO_2$ (95 atom% ^{15}N): C 50.85, H 9.39, N 12.66. Mass spectrum *m/e* 143 (5), 144 (95). By gas–liquid chromatography > 99% pure.

4. Discussion

The yields of L-α-aminobutyric acid (68%) L-methionine (41%) and L-valine (42%) obtained are less than the 80% yield of L-glutamic acid previously reported [11]. However, the method described is advantageous in that the product is easy to isolate in a pure state and contains an atom% ^{15}N-label which is equal to that of the [^{15}N]ammonium chloride used. Excess [^{15}N]ammonia could be recovered by distillation into concentrated HCl from the alkaline reaction mixture prior to column chromatography.

The reaction was terminated before completion to avoid the possibility of bacterial contamination. Yields of amino acid could presumably be increased by the addition of a bacteriocidal compound to the reaction mix, which would permit a longer safe reaction period.

We also think that the pH adjustments during the reaction, effected by the addition of 1 M KOH, probably caused denaturation of enzyme protein due to areas of high local pH and thus lowered the level of active enzyme. Adjustment of pH using 0.1 M KOH, perhaps associated with a pH-stat, is therefore likely to increase amino acid yields.

Using commercial biochemicals the reactions described appear comparatively expensive. However one major cost, that of the α-keto acids, can be avoided if the required-α-keto acids are synthesised by the reaction of either L- or D-amino acid oxidase with the appropriate amino acid [17–19]. If desired the amino acid oxidase could be used in an insoluble form [20,21].

Acknowledgements

The authors wish to thank Sir Ewart Jones for providing facilities for optical rotation and elemental analysis, Dr D. Harvey for performing mass spectroscopic analyses and Prochem. Ltd for donating the $^{15}NH_4Cl$.

References

[1] Aqvist, S. E. G. (1951) Acta Chem. Scand. 5, 1046–1064.
[2] Arnstein, H. R. V., Hunter, G. D., Muir, H. M. and Neuberger, A. (1952) J. Chem. Soc. 1329–1334.
[3] Araki, K., Takasawa, Y. and Nakajima, J. (1975) Agr. Biol. Chem. 39, 1193–1200.
[4] Tsuchida, T., Yoshinaga, F., Kubota, K. and Momose, H. (1975) Agr. Biol. Chem. 39, 1319–1322.
[5] Chibata, I., Tosa, T. and Sato, T. (1974) Appl. Microbiol. 27, 878–883.

[6] Eni, H., Nakazawa, H., Okumura, S. and Yamada, H. (1973) Agr. Biol. Chem. 37, 725–735.
[7] Sakurai, S. (1956) J. Biochem. Tokyo 43, 851–855.
[8] Sakurai, S. (1957) J. Biochem. Tokyo 44, 47–50.
[9] Yamada, S. and Sakurai, S. (1957) J. Biochem. Tokyo 44, 557–564.
[10] Sakurai, S. (1958) J. Biochem. Tokyo 45, 379–385.
[11] Greenaway, W. and Whatley, F. R. (1975) J. Labelled Cpds. 11, 395–400.
[12] Struck, J. and Sizer, I. W. (1960) Arch. Biochem. Biophys. 86, 260–266.
[13] Wiggert, B. O. and Cohen, P. P. (1965) J. Biol. Chem. 240, 4790–4792.
[14] Silverstein, E. and Subebele, G. (1974) Biochemistry 13, 1815–1818.
[15] King, K. S. and Cohen, P. P. (1975) Comp. Biochem. Physiol. 51B, 113–117.
[16] Bower, V. E. and Bates, R. G. (1955) J. Res. Natn. Bur. Stand. 55, 197–200.
[17] Meister, A. (1952) J. Biol. Chem. 197, 309–317.
[18] Meister, A. (1953) Biochem. Preps. 3, 66–70.
[19] Greenstein, J. P. and Winitz, M. (1961) in: Chemistry of the amino acids, 2, 1789–1793, John Wiley and Sons.
[20] Weetall, H. H. and Baum, G. (1970) Biotechnol. Bioengineer 12, 399–407.
[21] Tosa, T., Sano, R. and Chibata, I. (1974) Agr. Biol. Chem. 38, 1529–1534.