TENTATIVE IDENTIFICATION OF THE AMINO ACID THAT BINDS TYROSINE AS A SINGLE UNIT INTO A SOLUBLE BRAIN PROTEIN

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1. Introduction

A protein system that upon addition of ATP, Mg^{2+} and K^+ can incorporate tyrosine or phenylalanine as single units has been found in the soluble fraction of rat brain homogenate [1,2]. Some properties of the protein that incorporates these amino acids are similar to those generally considered distinctive of the microtubule proteins [3]. It was also reported that tyrosine & phenylalanine is bound to the carboxyl end of the protein [2], but the binding amino acid of the acceptor protein was not identified. In the work reported herein, radioactive peptides were isolated after partial acid hydrolysis of the labelled product containing [^{14}C] tyrosine (proteinyl-[^{14}C] tyrosine), in attempts to identify the dipeptide containing the labelled amino acid.

2. Materials and methods

2.1. *Materials*

α-L-Glutamyl-L-tyrosine was from Cyclo Chemical, Los Angeles, California. α-L-Aspartyl-L-tyrosine was a gift of Dr A. C. Paladini in whose laboratory was prepared by the method of solid phase synthesis [4], and was purified in our laboratory by TLC with ethanol-34% ammonium hydroxide (70:30, v/v) as solvent. Silica gel G was from E. Merck, Darmstadt, Germany.

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2.2. *Preparation of proteinyl-[^{14}C] tyrosine*

The soluble protein from rat brain homogenate was prepared as previously described [1] except that the soluble fraction was not passed through a column of Sephadex G-25. The incubation system contained, in 1 ml: 0.6 ml (6 mg of protein) of the soluble fraction from rat brain homogenate; 2.5 μmol of ATP; 30 μmol of KCl; 12.5 μmol of $MgCl_2$; 25 μmol of Tris-HCl buffer (pH 7.4) and 3.15 nmol (1.5 μCi) of [^{14}C] tyrosine. The incubation was at 37°C for 30 min. After cooling, [^{14}C] tyrosine and all other low molecular weight components of the incubation system were eliminated by passage through a column of Sephadex G-25. The total radioactivity incorporated into protein was approximately 400 000 cpm.

2.3. *Electrophoretic and chromatographic procedures*

Paper electrophoresis was carried out on Whatman 3MM at 10 V/cm during 5–6 hr. The solvent used was pyridine–acetic acid–water (10:0.4:90, by vol., pH 6.5).

For paper chromatography, Whatman 3MM was used and the solvent system was *n*-butanol–acetic acid–pyridine–water (30:6:20:24, by vol).

For TLC, silica gel plates, 0.25 mm thick were used and the solvent systems were: Solvent A: *n*-butanol–acetic acid–water (80:20:20, by vol); Solvent B: phenol–water (75:25, w/v); Solvent C: tert–butanol–formic acid–water (70:15:15, by vol), and Solvent D: ethanol–34% ammonium hydroxide (70:30, v/v).

Radioactive compounds on paper and silica gel plates were revealed by radioautography and amino acids and peptides by the ninhydrin reaction.

For ion exchange chromatography, a column

(0.9 X 6.0 cm) of Dowex 1X8-400 equilibrated with 3% pyridine was employed at room temperature with a flow rate of 15 ml/hr and fractions of 2 ml were collected. Aliquots of 0.2 ml were taken for radioactivity determinations. Discontinuous gradient elution was carried out by passing successively the following solutions: 3% pyridine, 11 ml; 0.1 M pyridine–acetic acid (pH 5.0), 40 ml; 0.5 M acetic acid, 20 ml; and 2 M acetic acid, 20 ml.

2.4. *Edman degradation*

This was carried out according to De Lange et al. [5]. After extraction of the anilinothiazolinones with butyl acetate, the radioactivity was measured in the aqueous and organic phases. In the cases in which radioactivity remained in the aqueous phase, a fraction of this material was utilized for electrophoretic and chromatographic analysis.

3. Results

A sample containing proteinyl-[^{14}C] tyrosine (5 mg of protein, 350 000 cpm) was hydrolyzed in 6 N HCl in an evacuated sealed glass tube for 20 min at 100°C.

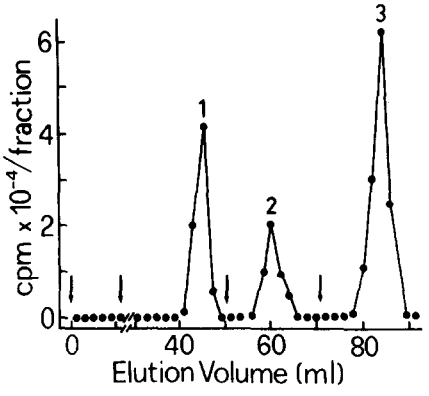

Fig.1. Chromatography on Dowex 1 of the partial acid hydrolysate of proteinyl-[^{14}C] tyrosine. The dried hydrolysate was redissolved in 1 ml of 3% pyridine and 300 000 cpm of this material were applied onto the Dowex 1 column. Elution and radioactivity determinations were as described in Materials and methods. The arrows in the figure mark changes of elution solvent.

The hydrolyzed material was chromatographed on a Dowex 1 column. Three radioactive peaks were obtained as shown in fig.1. The radioactive materials were dried separately in vacuum over NaOH. Paper electrophoresis, paper chromatography and TLC were used to ascertain the radioactive homogeneity of the material of each peak. The material from peak 1 (23% of the total radioactivity applied to the column) was identified as tyrosine by paper chromatography (R_f = 0.56) and TLC (Solvent A, R_f = 0.60; Solvent B, R_f = 0.45). The materials from peaks 2 and 3 (carrying respectively, 17 and 43% of the total radioactivity applied) were subjected to successive Edman degradations. For peak 2, more than 95% of its radioactivity was found in the organic phase in the second cycle, whereas for peak 3, three cycles were required for the radioactivity (more than 95% of its total) to appear in the organic phase. After a first degradation cycle the remaining radioactive material from peak 3 was found identical to peak 2 by paper electrophoresis and chromatography, and by TLC (Solvent A and B). After one degradation cycle, the radioactive material from peak 2 was identified as tyrosine. These results indicated that the material of peak 2 was a dipeptide and that of peak 3 a tripeptide comprising the dipeptide of peak 2 as a part of its composition. Furthermore, the electrophoretic behaviour of the radioactive dipeptide (peak 2) indicated that it contained an acidic amino acid; similar considerations led to the conclusion that the radioactive tripeptide (peak 3) contained two acidic amino acids.

The radioactive dipeptide was identified as α-L-glutamyl-L-[^{14}C] tyrosine. This was carried out by comparing the radioactive dipeptide with α-L-glutamyl-L-tyrosine and α-L-aspartyl-L-tyrosine. The dipeptide from proteinyl-[^{14}C] tyrosine cochromatographed with authentic α-L-glutamyl-L-tyrosine on paper (R_f = 0.54) and in TLC (Solvent A, R_f = 0.53; B, R_f = 0.20; C, R_f = 0.77 and D, R_f = 0.45). On the other hand, comparison of the dipeptide from proteinyl-[^{14}C] tyrosine with α-L-aspartyl-L-tyrosine showed that they moved differently in TLC (Solvent A, R_f of α-L-aspartyl-L-tyrosine = 0.46).

These results indicated that glutamic acid or glutamine (which would be totally converted to glutamic acid under the conditions of hydrolysis) is the COOH-terminal amino acid of the protein to which tyrosine is bound as a single amino acid.

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