RELEASE OF TYROSINE INCORPORATED AS A SINGLE UNIT INTO RAT BRAIN PROTEIN[1]

J. A. Rodríguez, C. A. Arce[2], H. S. Barra and R. Caputto

Departamento de Química Biológica, Facultad de Ciencias Químicas
Universidad Nacional de Córdoba, Córdoba. R. Argentina, South America

Received July 24, 1973

SUMMARY: L-Tyrosine incorporated as a single unit into the soluble protein of rat brain homogenate can be exchanged with free tyrosine, or released by the same enzymatic preparation. Whereas ATP is required for the incorporation reaction, ADP and inorganic orthophosphate (Pi) are required for the release reaction. A lesser activation, showing a lag period, of the release reaction by ATP or ADP free of Pi was observed. The cations Mg^{2+} and K^{+} are common requirements for both reactions.

INTRODUCTION

L-Tyrosine and L-phenylalanine are incorporated as single units into the soluble protein of rat brain homogenate by an enzymatic reaction that requires ATP, Mg^{2+} and K^{+} as cofactors (1, 2). Disc electrophoresis on dodecyl sulphate-polyacrylamide gel of the proteins after labeling with ^{14}C-tyrosine or ^{14}C-phenylalanine (and freed of unbound amino acid) showed about 24 bands stained by Coomassie brillant blue but only one was radioactive (this band contained about 95 per cent of the radioactivity recovered). Moreover, comparison of the electrophoretic patterns of preparations labeled with, respectively, ^{14}C-tyrosine and ^{14}C-phenylalanine showed the radioactivity in the same band for both preparations (unpublished results). The biological role of these reactions is not known but they may possibly be related to some specific function of the brain since the amounts of L-tyrosine or L-phenylalanine incorporated by a preparation from this organ were about 25- to 100-fold higher than those incorporated by comparable

1 This work was supported in part by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

2 Recipient of a scholarship from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

preparations from liver, kidney or thyroid (1, 2). Furthermore, tyrosine and phenylalanine reciprocally inhibit their respective incorporations (2) what suggests that these reactions may be of interest in relation to the effects produced by the accumulation of phenylalanine in patients affected of phenylketonuria.

The reaction mechanism of the incorporation of tyrosine and phenylalanine into brain protein differs from that of other amino acids also incorporated as single units, such as L-arginine by mammalian tissues (1, 3, 4) and L-leucine and L-phenylalanine by Escherichia coli (5). The main differences are that tRNA is not required for the incorporation of tyrosine or phenylalanine by brain preparation and that they are bound to the protein at a COOH-terminal position (2).

As an approach to the study of the mechanism of the reaction we were interested in obtaining reversion of these incorporations. In the present communication we report results that indicate that tyrosine incorporated into protein can be released and exchanged with free tyrosine. We also report the cofactors required for this release.

MATERIALS AND METHODS

Brains from albino rats of 30 days of age were used. Uniformly ^{14}C-labeled L-tyrosine was from New England Nuclear Corporation (Boston, Mass.), unlabeled L-tyrosine, Sephadex G-25-40 and pancreatic ribonuclease (Type III-A, protease free) were from Sigma Chemical Co. (St. Louis, Mo. USA).

Enzyme preparation and activity determination. Soluble fraction from rat brain homogenate was used. The details for the preparation of this fraction were previously described (1). When required, the preparation was freed of amino acids and all other cofactors of low molecular weight by passage through a column of Sephadex G-25-40 (6) equilibrated with a buffer solution identical to that used to prepare the homogenate. The incubation media will be given in detail for each experiment. The determination of ^{14}C-tyrosine incorporated into protein was as previously described (1).

RESULTS AND DISCUSSION

Exchange reaction. The enzymatic system that incorporates tyrosine as a single unit is very unstable; a supernatant fraction of a centrifugation at 100,000 g for 1 hour lost more than 80 per cent of its original activity after 24 hours of storage at 4°C. By adding glycerol (30 per cent final concentration) the system was stabilized, so that it lost less than 20 per cent of its activity after 24 hours at 4°C. By using the enzyme preparation stabilized with glycerol results were obtained which indicated that ^{14}C-tyrosine incorporated into protein during 30 minutes of incubation at 37°C, was substituted by unlabeled tyrosine (Figure 1, experiment A). That this was an exchange and not a

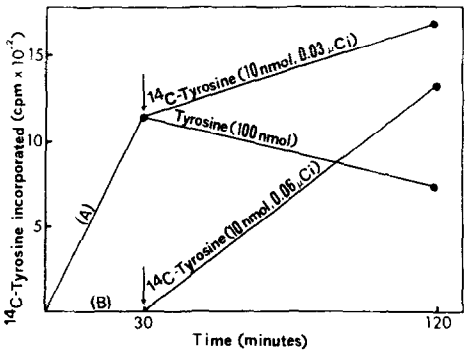

Figure 1. Exchange of protein-bound tyrosine with free tyrosine. The incubation systems (0.2 ml) contained: 0.5 μmol of ATP, 6 μmol of KCl, 2.5 μmol of $MgCl_2$, 5 μmol of tris-HCl buffer (pH 7.4), 0.1 ml (0.7 mg of protein) of 100.000 g supernatant fraction (containing 30 per cent of glycerol) from a rat brain homogenate and (A) 0.01 μmol (0.03 μCi) of ^{14}C-tyrosine or (B) 0.01 μmol of unlabeled tyrosine. After incubation of systems A and B for 30 minutes, labeled or unlabeled tyrosine (in 0.01 ml) was added as indicated and incubation was continued for another 90 minutes. The reactions were stopped by addition of 2 ml of 5 per cent (w/v) trichloroacetic acid, and the tubes were immersed in a water-bath at 90°C for 15 minutes. The suspensions were cooled and the precipitates collected on Millipore filter membranes and counted. Results are means of three experiments.

---

degradation of the labeled protein, was confirmed by substitution of unlabeled tyrosine by ^{14}C-tyrosine (Figure 1, experiment B). In this case the substitution was measured by actual binding of radioactivity from ^{14}C-tyrosine; the extent of the exchange reaction

calculated from the difference of incorporation of ^{14}C-tyrosine between experiment B and A (upper curve) during the period 30 - 120 minutes was approximately 70 per cent of the radioactivity incorporated at 30 minutes.

Release of ^{14}C-tyrosine. The role of ATP in the binding of tyrosine to brain protein has not been elucidated. Since tRNA was not required in this incorporation it appeared that the role of ATP was not identical to that played in the classical mechanism of protein biosynthesis. Furthermore, since ^{14}C-tyrosine was incorporated into a COOH-terminal position (2) it appeared that ATP was no required to activate the carboxyl group of the amino acid to be incorporated. A further step in the investigation of the role played by ATP in the binding reaction was the finding that ADP and inorganic orthophosphate (Pi) were the cofactors required for the release of tyrosine (Figure 2). If we assume that the forward and the backward reactions were carried out by the same enzyme the overall reaction is as follows:

(I) Protein + ATP + ^{14}C-tyrosine $\rightleftharpoons^{Mg^{2+},\ K^{+}}$ Proteinyl-^{14}C-tyrosine + ADP + Pi

A lesser effect, after a lag period, on the releasing of ^{14}C-tyrosine by ATP or ADP by itself, was observed (Figure 2). Since the preparation used was not purified these activations were probably due to the formation of ADP and Pi catalyzed by other enzymes present in the brain preparation.

Overall reactions similar to (I) have been described for γ-glutamylcysteine synthetase (7) and glutathione synthetase (8).

Identification of ^{14}C-tyrosine as the released product. For identification of the component released from the labeled protein, the following experiment was carried out: an enzyme preparation (0.15 ml) containing protein labeled with ^{14}C-tyrosine (7000 c.p.m.) and 30 per cent glycerol was mixed with ADP (0.1 μmol), Pi (0.2 μmol), KCl (6 μmol), $MgCl_2$ (2.5 μmol), RNase (60 μg) and tris-HCl buffer at pH 7.4 (5 μmol) in a total volume of 0.2 ml. After incubation at 37°C for 90 minutes the reaction was stopped

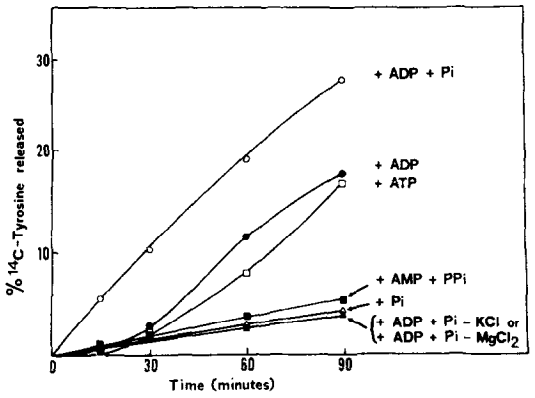

Figure 2. Requirements for the release of ^{14}C-tyrosine incorporated as a single unit into brain protein. The incubation system (0.2 ml) contained: 2000 c.p.m. of ^{14}C-tyrosine-labeled protein (for preparation see below), 5 µmol of tris-HCl buffer (pH 7.4), 6 µmol of KCl, 2.5 µmol of $MgCl_2$ and 0.1 ml (0.7 mg of protein) of 100.000 g supernatant fraction from a rat brain homogenate. The supernatant fraction had been passed through a column of Sephadex G-25-40 and then glycerol was added to 30 per cent final concentration. Where indicated 0.1 µmol of ADP, or ATP, or AMP or inorganic pyrophosphate (PPi) or 0.2 umol of inorganic orthophosphate (Pi) was added. The mixtures were incubated at 37°C and inactivated by addition of 1 ml of 6 per cent (w/v) trichloroacetic acid, and the tubes were immersed in a water-bath at 90°C for 15 minutes. The suspensions were cooled and centrifuged at 4000 rev./min for 15 minutes. The supernatant fluids were separated and counted. The amount of ^{14}C-tyrosine liberated from a control inactivated at time zero was discounted from each determination. The percentages were calculated in relation to the amount of ^{14}C-tyrosine-labeled protein found in the control at time zero. Results are means of two experiments.

^{14}C-Tyrosine-labeled protein was obtained by incubating in 1 ml: 2.5 µmol of ATP, 12.5 µmol of $MgCl_2$, 30 µmol of KCl, 840 pmol (0.4 µCi) of ^{14}C-tyrosine, 25 µmol of tris-HCl buffer (pH 7.4) and 0.8 ml (6 mg of protein) of 100.000 g supernatant fraction containing 30 per cent glycerol and 600 µg of pancreatic ribonuclease. After 15 minutes of incubation at 37°C, the preparation was cooled and 0.1 µmol of unlabeled tyrosine added; the mixture was passed through a column of Sephadex G-25-40 equilibrated with a buffer solution identical to that used to prepare the homogenate and then glycerol was added to 30 per cent final concentration. The excluded ^{14}C-tyrosine-labeled protein was immediately used.

---

by addition of 1 ml of hot 75 per cent (v/v) ethanol containing 40 µg of unlabeled L-tyrosine and centrifuged at 4000 rev./min for 15 minutes. The supernatant fluid (containing 2,300 c.p.m) was passed through a column (0.6 x 5 cm) of Dowex 50 H^+ and the column washed. More than 90 per cent of the radioactivity of the supernatant fluid was recovered with the fraction eluted with 1 M NH_4OH. This fraction was evaporated to dryness and

the remaining material was dissolved in water and subjected to thin-layer chromatography on silica gel G (Merck) using phenol-water (75:25, w/w) as solvent. Autoradiogram of the plate showed one spot (Rf = 0.47) which was in the position of authentic tyrosine.

ACKNOWLEDGEMENT: We wish to thank Prof. Alicia R. de Maccioni for criticism of the manuscript.

REFERENCES

1. Barra, H.S., Rodríguez, J.A., Arce, C.A., and Caputto, R., J.Neurochem. 20, 97 (1973).
2. Barra, H.S., Arce, C.A., Rodríguez, J.A., and Caputto, R., J.Neurochem. In press (1973).
3. Soffer, R.L., Biochem.Biophys.Acta, 155, 228 (1968).
4. Soffer, R.L., and Horinishi, H., J.Mol.Biol., 43, 163 (1969).
5. Momose, K., and Kaji, A., J.Biol.Chem., 241, 3294 (1966).
6. Barra, H.S., Uñates, L.E., Sayavedra, M.S., and Caputto, R., J.Neurochem. 19, 2289 (1972).
7. Orlowski, M., and Meister, A., J.Biol.Chem., 246, 7095 (1971).
8. Snoke, J.E., and Bloch, K., J.Biol.Chem., 213, 825 (1955).