RELEASE OF TYROSINE FROM TYROSINATED TUBULIN. SOME COMMON FACTORS THAT AFFECT THIS PROCESS AND THE ASSEMBLY OF TUBULIN

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1. Introduction

An enzyme system present in the soluble fraction of rat brain homogenate catalyzes the incorporation of tyrosine into the carboxyl end of the α-subunit of tubulin [1,2]. The system does not require nucleic acids [3]. Results from studies on the incorporation of [^{14}C] tyrosine into brain tubulin in animals whose protein synthesis was inhibited by cycloheximide indicated that a similar system operates in vivo [4].

The present work deals with an activity found in the soluble fraction of rat brain that determines the release of the C-terminal tyrosine from tyrosinated tubulin. Every previously known inhibitor or activator of assembly of tubulin we tested also affected the release of tyrosine when assayed in similar conditions. The relationship of the activity reported here with a similar one previously described [5] for which was claimed that ADP and P_i were required is discussed.

2. Experimental Procedure

2.1. *Preparation of tubulinyl-[^{14}C]tyrosine*

Rat brain tissue was homogenized in 1 vol. of 100 mM PIPES (piperazine-*N,N'*-bis(2-ethane sulfonic acid)) buffer, pH 6.8, containing 1 mM EGTA (ethyl-eneglycol-bis (β-aminoethyl ether)*N,N'*-tetraacetic acid). After centrifugation at 100 000 × *g* for 1 h the supernatant fluid was passed through a column (1 × 25 cm) of Sephadex G-25 equilibrated with a buffer solution identical to that used to prepare the homogenate. For incorporation of [^{14}C]tyrosine into tubulin the incubation mixture contained in 1 ml: 2.5 μmol ATP, 12.5 μmol $MgCl_2$, 250 μmol KCl, 0.1 μmol [^{14}C]tyrosine (6 μCi/μmol) and 0.8 ml of brain preparation (approx. 10 mg protein). After 20 min at 37°C the incubation mixture was cooled to 4°C and passed through a column (1 × 25 cm) of Sephadex G-25 equilibrated with PIPES–EGTA buffer.

Protein was determined according to Lowry et al. [6].

2.2. *Determination of [^{14}C]tyrosine release and tubulin assembly*

To measure the release of [^{14}C]tyrosine, glycerol (4 M final concentration) was added to the tubulinyl-[^{14}C]tyrosine preparation and the mixture incubated at 37°C for 90 min. For control purpose a similar mixture was maintained at 4°C. The reaction was stopped by addition of 2 ml 5% (w/v) trichloroacetic acid to 0.15 ml of the incubation mixture and the inactivated suspension kept at 90°C for 15 min. After centrifugation, the [^{14}C]tyrosine released was measured in the supernatant solution by counting in a scintillation spectrometer. The assembly of tubulin was estimated by turbidity measurements at 350 nm [7] in the same incubation system and conditions used for tyrosine release.

2.3. *Polyacrylamide gel electrophoresis*

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% acrylamide, 0.13% bisacrylamide) was performed as described by Weber and Osborn [8]. Staining, destaining and radioactivity measurements of the gels were carried out as previously described [1].

Table 1
Release of [^{14}C]tyrosine and assembly of tubulin

Addition (mM)	[^{14}C]Tyrosine released (%)	Extent of assembly ΔA_{350}
None	24	0.19
$MgCl_2$ (0.5)	36	0.34
GTP (1)	10	0.22
GTP (1) + $MgCl_2$ (0.5)	60	0.46
GTP (1) + $MgCl_2$ (0.5) + colchicine (0.2)	15	n.d.
GTP (1) + $MgCl_2$ (0.5) + KCl (250)	2	0.02
GTP (1) + $MgCl_2$ (0.5) + NaCl (250)	2	0.03
ADP (1) + P_i (0.2)	8	0.14
ADP (1) + $MgCl_2$ (0.5)	57	0.35
ADP (1) + P_i (0.2) + $MgCl_2$ (0.5)	57	0.38
$MgCl_2$ (12)	6	0.16
ADP (1) + P_i (0.2) + $MgCl_2$ (12)	36	0.25
ADP (1) + P_i (0.2) + $MgCl_2$ (0.5) + colchicine	21	n.d.

n.d. = not determined

The assays were carried out as described under Experimental Procedure, except that where indicated, the corresponding compound(s) added to the incubation mixture in 0.1 vol. of PIPES–EGTA buffer.

3. Results

3.1. *Conditions for tyrosine release*

When a labelled tyrosinated tubulin preparation, obtained as described above, was incubated at 37°C [^{14}C]tyrosine was released (table 1). Addition of glycerol (4 M) did not modify the release; however, to test the capacity of detyrosinated tubulin to reincorporate [^{14}C]tyrosine, glycerol was introduced in most of our experiments to stabilize the activity of the incorporating system [5]. $MgCl_2$ at 0.5 mM slightly activated the release but at concentrations higher than 10 mM produced a definite inhibition. GTP (1 mM) inhibited when added alone but when GTP (1 mM) and $MgCl_2$ (0.5 mM) were simultaneously present, the highest rate of release was obtained. Addition of ADP (1 mM) plus P_i (0.2 mM) also inhibited the release but activated in the presence of 0.5 mM $MgCl_2$. Furthermore, addition of ADP (1 mM) plus P_i (0.2 mM) counteracted the inhibition brought about by 12 mM $MgCl_2$. High salt concentrations (NaCl or KCl, 250 mM) almost completely stopped the release of [^{14}C]tyrosine. Colchicine (0.2 mM) in the presence of either 1 mM ADP plus 0.2 mM P_i plus 0.5 mM $MgCl_2$ or 1 mM GTP plus 0.5 mM $MgCl_2$ produced a significant inhibition of the release.

3.2. *The reaction products*

Proteolytic enzymes are present in the brain fluid that may conceivably degrade tubulin releasing tyrosine-containing peptides which would not be precipitated by trichloroacetic acid. For this reason it was investigated whether the products of the reaction were [^{14}C]tyrosine and detyrosinated tubulin.

For identification of the labelled component(s) released from labelled tubulin, the reaction was stopped by addition of 2 vol. of ethanol. After centrifugation, a portion of the supernatant fraction was subjected to thin-layer chromatography on Silica gel G after addition of 30 μg of unlabelled L-tyrosine. *n*-Butanol/acetic acid/water (80:20:20, by vol.) was used as solvent system. In the radioautogram most of the radioactivity was found exactly superimposable with the ninhydrin spot of L-tyrosine. After scraping it was found that the spot carried 90% of the radioactivity on the plate.

The recovery of detyrosinated tubulin after the release of tyrosine was ascertained by reincorporation of [^{14}C]tyrosine. Figure 1 shows that the amount of [^{14}C]tyrosine that entered after the releasing reaction was similar to the amount previously released. Moreover, only one labelled product was found on SDS–polyacrylamide gel electrophoresis after reincorpora-

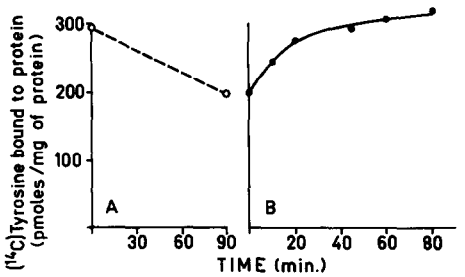

Fig.1. Capability of detyrosinated tubulin to reincorporate [^{14}C]tyrosine. The release of [^{14}C]tyrosine from tubulinyl-[^{14}C]tyrosine (A) was carried out as described under Experimental Procedure except that 0.5 mM $MgCl_2$ was added to the incubation mixture. For control purpose a similar mixture was maintained at 4°C (or incubated at 37°C but with 250 mM KCl added to inhibit the release of [^{14}C] tyrosine (see Table 1)). At the end of the incubation period the amount of [^{14}C]tyrosine released was tested and the capability of the detyrosinated tubulin to reincorporate [^{14}C]tyrosine was measured at different periods of time after the addition of 2.5 mM ATP, 12.5 mM $MgCl_2$, 250 mM KCl and 0.1 mM of [^{14}C]tyrosine (6 μCi/μmol). Values plotted (B) are after discounting the incorporations obtained in the control.

tion of [^{14}C]tyrosine into previously detyrosinated unlabelled tubulinyl-tyrosine (fig.2).

3.3. *Release of tyrosine and assembly of tubulin*

It became apparent that some of the factors described as influencing the assembly of tubulin also influenced the release of tyrosine from tyrosinated tubulin. Table 1 shows that the addition to the incubation system of 1 mM GTP and 0.5 mM $MgCl_2$ which are known to promote maximal tubulin assembly also produced maximal release of tyrosine. Withdrawal of GTP, $MgCl_2$ or both, decreased the release and the assembly, although not in the same proportion. Colchicine, a well known inhibitor of tubulin assembly in brain extracts [9,10] inhibited the release of tyrosine. High concentration of KCl or NaCl (250 mM) also known as inhibitors of assembly [11] almost completely stopped the release and the assembly.

ADP also promotes the assembly of tubulin [7]. A system containing 1 mM ADP plus 0.2 mM P_i plus 0.5 mM $MgCl_2$ activated the release and the assembly processes. ADP (1 mM) plus P_i (0.2 mM) reverted the inhibition brought about by 12 mM $MgCl_2$ on release and assembly.

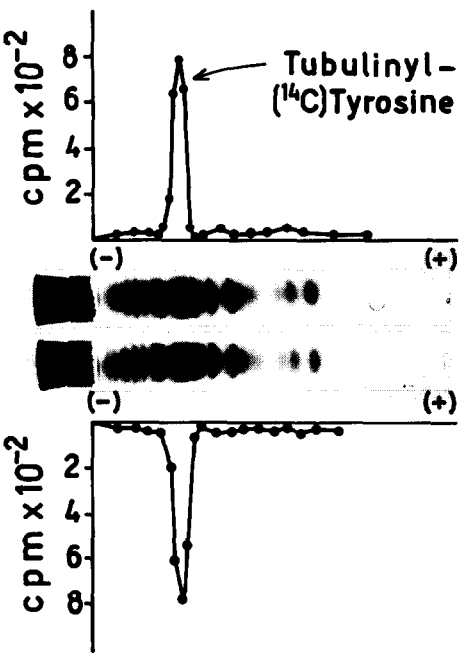

Fig.2. Sodium dodecyl sulfate–polyacrylamide electrophoresis. The conditions for electrophoresis and radioactivity measurements were as described under Experimental Procedure. Upper part: protein and radioactivity patterns of a preparation of tubulinyl-[^{14}C]tyrosine obtained as described under Experimental Procedure. Lower part: a preparation of unlabelled tubulinyl-tyrosine that was subjected to the release reaction during 90 min and then to incorporation of [^{14}C]tyrosine during 40 min in the conditions described in fig.1. The protein and radioactivity patterns shown correspond to this last material.

Starting with tyrosinated tubulin we were unable to obtain substantial assembly without release of tyrosine but could obtain release of tyrosine, not accompanied by detectable assembly. This was obtained when the reaction was carried out at pH 7.34 (instead of pH 6.8) in a system containing 1 mM GTP and 0.5 mM $MgCl_2$.

4. Discussion

The enzyme activity described herein is tentatively considered as a carboxypeptidase. We have not as yet determined whether it is specific for tyrosine or if the specificity is only apparent and due to a possible inability of the enzyme to remove glutamic acid that

follows the tyrosine residue in tyrosinated tubulin [12]. Neither have we definite information on whether the activity we are reporting is specific for tubulin. However, comparison of the gel electrophoretogram of tubulinyl-[^{14}C]tyrosine preparations, obtained before and after the preparations were subjected to a sequence of release and reincorporation of [^{14}C]tyrosine, did not show substantial alterations.

The notion that in tubulin the release affects mainly or exclusively tyrosine is supported by the following observations: ninety per cent of the radioactivity found in the products of the releasing reaction was identified as [^{14}C]tyrosine by thin-layer chromatography; no di- or tripeptide containing [^{14}C]tyrosine was observed. The amount of [^{14}C]tyrosine incorporated into a detyrosinated tubulin preparation was practically identical to that of the previously released tyrosine. The labelled protein obtained migrated on polyacrylamide electrophoresis as tubulinyl-tyrosine. These results showed that tubulin was not destroyed to a point that it lost its capacity to incorporate tyrosine or changed its electrophoretic properties. They indicate that tyrosine is the only amino acid released from tubulin during the reaction.

The relation of this enzyme activity with one previously described as requiring ADP and P_i [5] is not clear at present. Raybin and Flavin [2,13] have confirmed the existence of the enzyme that requires ADP and P_i besides $MgCl_2$ and separated the enzyme from its substrate. However, we have not been able to establish clearly that the requirement of ADP plus P_i is not to revert the inhibition brought about by 12 mM $MgCl_2$ on the activity here described. This concentration of $MgCl_2$ was the one used in our original experiments.

The rough parallelism observed between the releasing and assembling processes may indicate coincidental requirements related to the structure of tubulin, and not a link between both processes. On the other hand, excess tyrosine may bring about similar conditions to those found in the presence of inhibitors. Tubulin assembly could perhaps be obtained in the absence of a certain minimum level of detyrosinated tubulin. If this were the case, the regulation of the tyrosinating and detyrosinating activities could be significant with respect to the functioning of tubulin.

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