Association of tubulinyl-tyrosine carboxypeptidase with microtubules

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1. INTRODUCTION

Tubulin can be modified by the enzymatic incorporation or release of a tyrosine residue at the COOH-terminus of the α-chain. At least two enzymes are involved in this modification. The incorporation of tyrosine is catalyzed by the ATP-dependent enzyme tubulin.tyrosine ligase (TTLase) which seems to be specific for α-tubulin [1–3]. In the presence of ADP and P_i this enzyme can release the incorporated amino acid [4,5]. The COOH-terminal tyrosine of tubulin can also be removed by another enzyme, tubulinyl-tyrosine carboxypeptidase (TTCPase) which has been partially purified from brain tissue [6,7]. It has been reported that non-assembled tubulin is the substrate for the TTLase [8] whereas the TTCPase acts preferentially on intact microtubules [9,10]. Here, we describe the association of TTCPase with microtubules throughout successive cycles of assembly–disassembly.

Abbreviations: TTLase, tubulin.tyrosine ligase; TTCPase, tubulinyl-tyrosine carboxypeptidase; Pipes, piperazine-*N,N'*-bis(2-ethane sulfonic acid); Mes, 2(*N*-morpholino)ethane sulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetraacetic acid

2. MATERIALS AND METHODS

2.1. *Materials*

L-[U-^{14}C]Tyrosine (spec. act. 450 μCi/μmol) was obtained from New England Nuclear. Pipes, Mes, EGTA, GTP and Sephadex G-25 (20–80 μm) were purchased from Sigma (St Louis MO).

2.2. *Preparation of [^{14}C]tyrosinated microtubule protein by assembly–disassembly*

Brains from 30- to 60-day-old rats were homogenized in 1 vol. Pipes buffer (0.1 M Pipes adjusted with KOH to pH 6.7, containing 1 mM EGTA). The homogenate was centrifuged at 100000 × *g* for 1 h at 2–4°C and the supernatant solution was collected. For the incorporation of [^{14}C]tyrosine into tubulin, the incubation mixture contained, per ml: 0.9 ml soluble brain extract, 250 μmol KCl, 2.5 μmol ATP, 12.5 μmol $MgCl_2$ and 2.2–4.4 nmol (1–2 μCi) [^{14}C]tyrosine. After incubation for 20 min at 37°C the mixture was cooled and passed through a column of Sephadex G-25 equilibrated with Pipes buffer to eliminate unbound [^{14}C]tyrosine. The excluded material was mixed with 1 vol. non-labeled brain extract and after addition of glycerol (40% final conc.), GTP (1 mM) and $MgCl_2$ (0.5 mM), the mixture was incubated at 37°C for 30 min and then centrifuged at 100000 × *g* for 30 min at 27°C (warm centrifuga-

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tion) to sediment the microtubule fraction. The resulting post-assembly supernatant fraction was designated S_1. The pellet was resuspended in Mes buffer (0.1 M Mes adjusted with KOH to pH 6.7, containing 1 mM EGTA) to give ~3 mg protein/ml. After 30 min at 0°C the suspension was centrifuged at 100000 × *g* for 10 min at 2–4°C. The supernatant solution (microtubule protein purified by one cycle) was designated MTP(× 1). Subsequent assembly–disassembly cycles were carried out in Mes buffer in the presence of 1 mM GTP and 0.5 mM $MgCl_2$. The incubation time for assembly was 15 min and the warm centrifugation to separate the assembled from the non-assembled fraction was at 100000 × *g* for 10 min. The purified microtubule protein preparations MTP(× 1), MTP(× 2), and so forth were immediately used; × 1, × 2, indicate the number of the assembly–disassembly cycles. The post-assembly supernatant fractions obtained after warm centrifugation were designated S_1, S_2, etc.

2.3. *Determination of the [^{14}C]radioactivity released from tubulinyl-[^{14}C]tyrosine*

Unless otherwise specified, the protein preparations containing tubulinyl-[^{14}C]tyrosine were incubated at 37°C for 60 min in the presence of 1 mM GTP and 0.5 mM $MgCl_2$. After the incubation period the radioactivity bound to the trichloroacetic acid insoluble material was measured as in [1]. The radioactivity released was calculated as the difference between a zero time control and the experimental tube. All determinations were done in duplicate.

2.4. *Determination of tubulin–tyrosine ligase activity*

The incubation mixture contained in 0.2 ml final vol.: 0.15 ml tubulin preparation, 0.5 μmol ATP, 2.5 μmol $MgCl_2$ and 10 nmol (0.15 μCi) [^{14}C]tyrosine. The incubation was at 37°C for 5 min. The reaction was stopped by the addition of 2 ml 5% trichloroacetic acid. The radioactivity incorporated into protein was measured as in [1].

2.5. *Polyacrylamide gel electrophoresis*

The continuous sodium dodecyl sulfate gel system described in [11] was used. The gels (7.5% acrylamide, 0.097% bisacrylamide) were stained with Coomassie brilliant blue and destained by diffusion. The radioactivity in the gels was determined in 2-mm slices as in [2].

2.6. *Thin-layer chromatography*

Bidimensional thin-layer chromatography on silica gel G (Merck) was developed by using *n*-butanol/acetic acid/water (80:20:20, by vol.) for the first run and phenol/water (75:25, w/w) for the second run. The plate was dried and exposed to 3M-X-ray film. The position of authentic tyrosine was revealed by the ninhydrin method.

2.7. *Protein determination*

Proteins were determined by the method in [12].

3. RESULTS AND DISCUSSION

[^{14}C]Tyrosinated MTP preparations obtained after 1–5 cycles of assembly–disassembly as in section 2 were incubated at 37°C for 60 min in the presence of 1 mM GTP and 0.5 mM $MgCl_2$ and the radioactivity released was measured. Under these conditions all of the MTP preparations showed to contain a significant amount of TTCPase activity, indicating that the enzyme remained associated with microtubules during the assembly–disassembly procedure. The radioactivity released from each one of the MTP preparations ranged from 30 to 60%. Results from a representative experiment are shown in table 1. The time course of the releasing reaction and the dependence with protein concentration were studied. Results from an experiment carried out with a MTP(× 3) preparation are shown in fig.1. Similar results were obtained with MTP(× 1, × 2, × 4 and × 5) preparations.

The possibility that the release of radioactivity from [^{14}C]tyrosinated MTP preparations was due to the reversal of the TTLase reaction was ruled out; we did not detect the presence of TTLase in the MTP preparations after the second cycle of assembly–disassembly, as measured by the capacity of these preparations to catalyze the incorporation of [^{14}C]tyrosine. This confirmed previous results [4] about the distribution of TTLase between the assembled and non-assembled fraction. Furthermore, the addition of ADP, inorganic phosphate and $MgCl_2$ at optimal concentrations for the releasing activity of TTLase, inhibited

Table 1

Release of [^{14}C]tyrosine from [^{14}C]tyrosinated tubulin preparations obtained after 1–5 cycles of assembly–disassembly

[^{14}C]Tyrosinated tubulin preparation	[^{14}C]Tyrosine released (%)
S_1	8
MTP(× 1)	43
S_2	4
MTP(× 2)	40
S_3	2
MTP(× 3)	37
S_4	4
MTP(× 4)	31
S_5	4
MTP(× 5)	32

Assay conditions were as described in section 2. Protein in the incubation mixtures ranged from 2.8–3.2 mg/ml for the microtubule protein (MTP) preparations, and from 0.35–0.45 mg/ml for the post-assembly supernatant fractions (S)

rather than stimulated the release of radioactivity (not shown).

It is known that the COOH-terminal tyrosine can be released from tubulin by TTCPase without any apparent further digestion of tubulin [13]. To ascertain whether the enzymatic activity associated with microtubules was due to TTCPase or to an endopeptidase, the labeled materials were characterized after the incubation period. For this purpose a [^{14}C]tyrosinated MTP(× 3) preparation was analyzed by polyacrylamide gel electrophoresis before and after incubation in the conditions of the experiment shown in table 1. In these conditions about 40% of the radioactivity was released. As can be seen in fig.2 the protein pattern was not altered by the incubation, and the recovered radioactivity in the gel was detected only in a single protein band corresponding to [^{14}C]tyrosinated tubulin. In another experiment, after the incubation period, the protein fraction was precipitated by the addition of 2 vol. ethanol

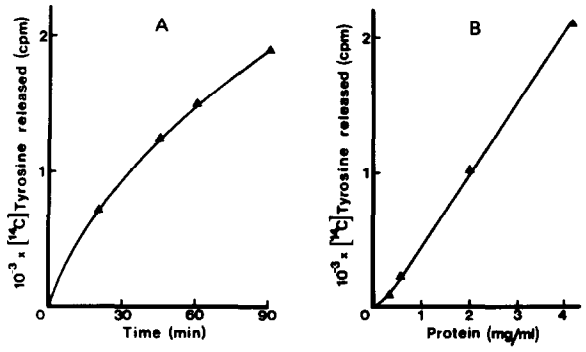

Fig.1. Release of [^{14}C]tyrosine as a function of the incubation time (A) and protein concentration (B). Assay conditions were as in section 2; in these determinations a [^{14}C]tyrosinated MTP (× 3) preparation was used. In (A), protein was 3 mg/ml.

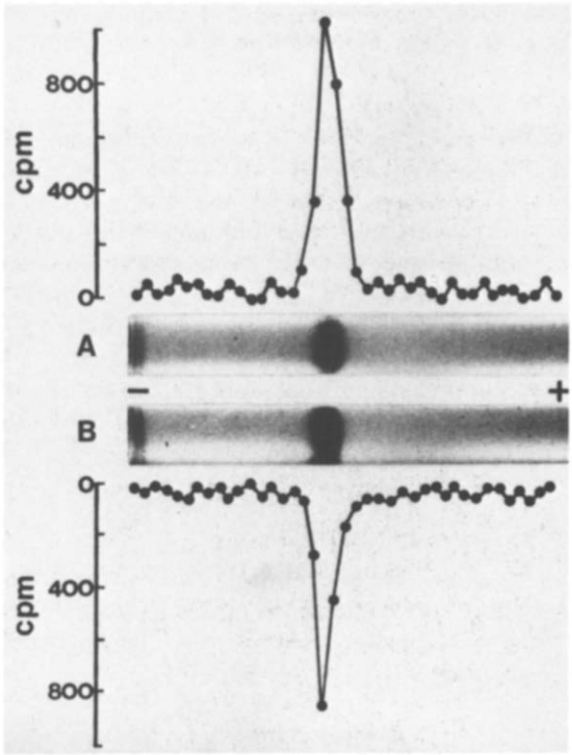

Fig.2. Effect of the incubation under releasing conditions on the integrity of tubulinyl-[^{14}C]tyrosine. A [^{14}C]tyrosinated MTP(× 3) preparation was incubated in the presence of 1 mM GTP and 0.5 mM $MgCl_2$ for 60 min at 37°C. Under these conditions 40% of the radioactivity was released. Aliquots (50 μg protein) were removed before (A) and after (B) incubation and subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis. The protein and radioactivity pattern were determined as in section 2.

at 90°C and the soluble fraction was dried and subjected to bidimensional thin-layer chromatography (see section 2). [^{14}C]Tyrosine was the only radioactive spot found. These results indicate that the release of the radioactivity from tubulinyl-[^{14}C]tyrosine was not due to an endopeptidase activity but to TTCPase. This enzyme may be considered as a microtubule-associated protein since it remained associated with microtubules during 5 cycles of assembly–disassembly.

The post-assembly supernatant fractions obtained during the successive cycles of assembly–disassembly were also incubated at 37°C for 60 min to measure the release of [^{14}C]tyrosine from the tubulinyl-[^{14}C]tyrosine molecules that remained in the non-assembled pool. As shown in table 1, the values found were practically negligible in all of the cases with the exception of S_1. Protein in the incubation mixtures ranged from 0.35–0.45 mg/ml.

It has been reported [14] that a rapid turnover of the COOH-terminal tyrosine residue, in living muscle cells in culture, is dependent upon the presence of intact microtubules. Furthermore, microtubules seem to be better substrates than tubulin dimers for TTCPase [9,10]. These observations are in good accordance with our finding that TTCPase associates with microtubules. This association could be an important event in the regulation of the tyrosination state of the COOH-terminus of α-tubulin.

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