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TYROSINATION STATE OF FREE TUBULIN SUBUNITS AND TUBULIN DISASSEMBLED FROM MICROTUBULES OF RAT BRAIN TISSUE

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Abstract: The content of endogenous COOH-terminal tyrosine in unpolymerized tubulin subunits from the brain extracts of new-born rats was twice as high as that of tubulin isolated from disassembled native microtubules. It was also observed that the proportion of free sites in tubulin from in vitro incorporation of tyrosine at the COOH-terminus in unpolymerized protein was about one-half of that obtained for the protein from disassembled microtubules. In the extracts of young adult rats the value for the endogenous COOH-terminal tyrosine content in unpolymerized subunits decreased and almost equaled the value for the polymerized tubulin which remained virtually constant.

Cytoplasmic tubulin has been found to undergo a postsynthetic addition of an aromatic residue, mainly tyrosine, into the COOH-terminal end of the α chain of a proportion of the molecules (1,3,11). The reaction is catalyzed by an ATP-dependent enzyme which seems to be highly specific for α tubulin (10). Because of its unique features, the reaction of incorporation of tyrosine has been considered to play a regulatory role for the activity or function of tubulin. However, several pieces of evidence have indicated so far that the reaction of polymerization in vitro is not affected by the presence or the absence of tyrosine at the COOH-terminal end (2,9). This observation does not rule out the possibility that in the cell the tyrosination of the α subunit could participate in the control mechanism of the relative distribution of tubulin between the polymer and monomer states. Conditions in vivo could be such that tubulin molecules are discriminated by the configuration of the COOH-terminal portion which could be ultimately determined by the nature of the residue at that end. We have used a procedure to stabilize native microtubules from brain tissue and to separate them from unpolymerized subunits. Our purpose was to isolate tubulin from both conditions and analyze the state of the COOH-terminus by assaying tyrosine after

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release with carboxypeptidase A. The data reported here indicate that although tubulin from the two states of assembly contained COOH-terminal tyrosine, there were quantitative differences. We have also observed that the availability of sites for *in vitro* tyrosination showed quantitative differences as well for the two forms of tubulin in such a fashion that the higher the endogenous content of COOH-terminal tyrosine, the lower the capacity to incorporate the amino acid *in vitro*.

Materials and Methods

Native microtubules and unpolymerized subunits were prepared from brains of male albino rats (Holtzman Co., Madison, WI) according to the procedure of Pipeleers et al. (1977) with minor changes. The tissue (5 g) was homogenized with 10 vol of a stabilizing solution containing 50% glycerol (Sigma), 5% dimethyl sulfoxide (Fisher), 0.5 mM GTP (Aldrich), 0.5 mM $MgCl_2$, 0.5 mM EGTA, 10 mM sodium phosphate buffer, pH 7.2 and 0.1 mM $CuCl_2$. The $CuCl_2$, which did not affect the recovery of microtubules, was included to prevent the action of an endogenous carboxypeptidase activity reported in brain (4). The homogenization was performed in a 50-ml teflon-in-glass homogenizer with a difference in diameter between pestle and tube of 0.23-0.25 mm, at 850 rpm for 5 up-and-down strokes. The homogenate was centrifuged at 1000 x g for 10 min to remove large debris, and then the supernatant was centrifuged at 105,000 x g for 45 min. The resulting pellet and supernatant contained the stabilized microtubules (polymer fraction) and unpolymerized subunits (monomer fraction), respectively. The supernatant fraction was removed and the pellet was resuspended in an equal volume of a solution containing 0.1 M Pipes, pH 6.94, 0.1 mM $MgCl_2$ and 0.1 mM GTP. All operations up to this point were carried out at room temperature (23°C).

The polymer fraction was made 0.25 M in KCl, kept in ice for 30 min to depolymerize the microtubules, centrifuged at 105,000 x g for 30 min at 3°C and the supernatant containing the subunits from the disassembled microtubules was removed. The monomer fraction was applied to a Sephadex G-50 column which was equilibrated and eluted at 4° with the same solution as above to change the sample to the same buffer as the pellet. The voided fraction was eluted and made 0.25 M in KCl to correspond to the treatment of the pellet.

Tubulin was isolated from both polymer and monomer fractions by ion-exchange chromotography on DEAE-Sephadex and analyzed for its content of COOH-terminal tyrosine by the fluorimetric technique as described previously (11). Anlaytical SDS-electrophoresis of the tubulin fraction showed a degree of purity for the protein in the range of 95 to 99%.

Maximal capacity for incorporation of ^{14}C-tyrosine was measured in the two fractions as obtained just before the ion-exchange chromatography step. The conditions for the assay were as described previously (5) except for the addition of partially purified porcine tubulin-tyrosine ligase to insure maximal incorporation. Ligase was purified from pig brain tissue approximately 15-fold according to the procedure of Raybin and Flavin (1977).

Samples taken for electron microscopy were negatively stained as described by Borisy et al. (1974).

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Results and Discussion

We used the conditions described by Pipeleers et al. (1977) to preserve the native microtubules of rat brain tissue. Microtubules and unpolymerized subunits in extracts of the tissue were separated by differential centrifugation. Electron microscopy of the polymer fraction showed a large number of native microtubules. These slowly disassembled in the cold but depolymerized rapidly after addition of 0.25 M KCl. Tubulin was isolated by DEAE-chromatography from the depolymerized native microtubules and from the monomer fraction (see Materials and Methods) and the degree of purification was 95-99%. This material was used for direct determinations of the endogenous content of COOH-terminal tyrosine assayed after release by carboxypeptidase A digestion. Animals of 1, 7 and 60 days of age were used and the amount of protein (DEAE-tubulin) recovered from each fraction is compared in Table 1. Under the conditions used approximately 60% of the protein was recovered in the form of pelletable microtubules in both new-born and young adult rats. The total amount of tubulin recovered in the two fractions is comparable to the value calculated from data reported by Arce et al. (1975).

The result of the analysis of DEAE-tubulin for its content of COOH-terminal tyrosine by spectrofluorimetry of the amino acid released by carboxypeptidase A digestion is shown in Fig. 1. The molar ratio of tyrosine bound to tubulin is shown for the three age points studied. Although tubulin from both polymer and monomer fractions contained COOH-terminal tyrosine, quantitative differences were observed. In one-week-old rats the proportion of tyrosinated molecules of non-assembled tubulin was 52% and the one for the protein purified from disassembled microtubules was 26%. The same relative difference was found for the material obtained from one-day-old rats although the absolute values were slightly lower. Measurements in the protein from young adult rats indicated that the proportion of tyrosinated

TABLE I

Recovery of tubulin from rat brain tissue

Age	Monomer	Polymer	% Polymer
days	mg/g of wet tissue		
1	1.06	1.56	59
7	0.98	1.64	65
60	0.64	1.12	63

Tubulin from monomer and polymer fractions was isolated by ion-exchange chromatography as described previously (11). Protein concentration was measured in the dialyzed eluates from the DEAE-columns. The degree of purity was determined by analytical SDS-electrophoresis (see Materials and Methods)

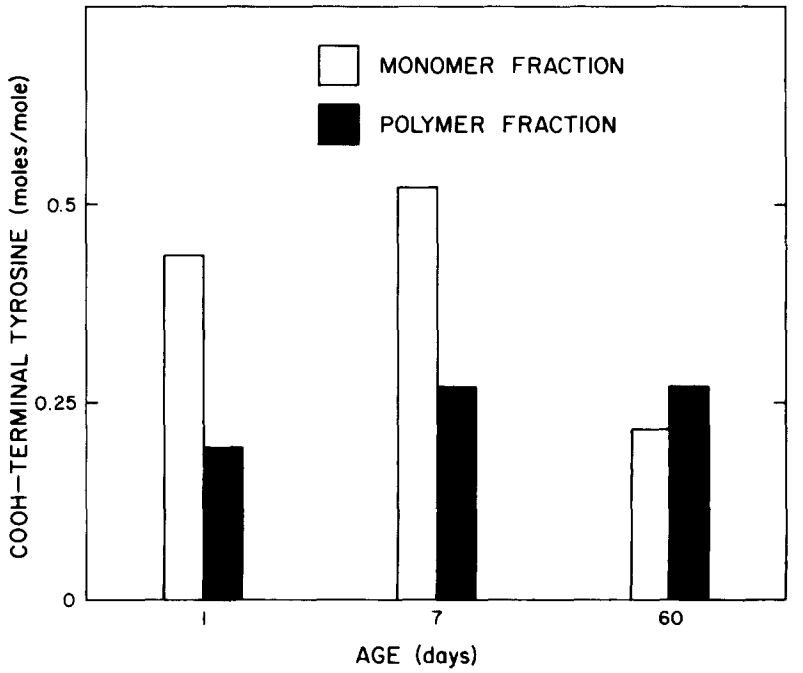

Figure 1. Histogram representing the relative content of COOH-terminal tyrosine in tubulin from the monomer and polymer fractions of rat brain. Tubulin from the fractions prepared as described under Material and Methods was partially digested with carboxypeptidase A and the tyrosine released was assayed fluorimetrically as described elsewhere (11). Molar ratios were calculated assuming a molecular weight for tubulin dimer of 110,000 daltons.

free tubulin had decreased to the level corresponding to microtubular tubulin which remained almost unchanged.

Several attempts have been made to correlate the state of tyrosination of the COOH-terminus of α tubulin and the capacity of the protein to poly-

merize in vitro. Tubulin seems to polymerize to the same extent with or without COOH-terminal tyrosine in in vitro conditions (2,9). Nevertheless, the conditions of the experiments in vitro may not be directly applicable to the conditions obtaining in vivo. The distribution of tubulin between the two states of assembly may depend on a number of different factors which might not be equivalent in the in vitro experiments. We have now carried out direct determinations of the extent of tyrosination in tubulin isolated from the two states of polymerization. Our findings indicate that the level of tyrosination of unassembled tubulin in extracts of developing brain is double that of tubulin from native microtubules, but that the levels of tyrosination of tubulin of the two fractions in young adult rats are almost equal. It is noteworthy that the change in the state of tyrosination of unassembled tubulin with the age of animals is analogous to that reported previously for cytoplasmic chick brain tubulin (11). The period of maximal changes in the brain of the chick embryo discussed in that report seems to occur in the rat a few days after the birth.

Tubulin from the stages of assembly was also compared for its capacity to incorporate ^{14}C-tyrosine in vitro. The assay was performed in the crude material after the differential centrifugation and resuspension and desalting of pellet and supernate, respectively. Partially purified porcine tubulin-tyrosine ligase (according to (10)) was used in order to insure maximal incorporation of the amino acid. The time-course of the incorporation reaction in both materials is shown in Fig. 2. The kinetic pattern was the same for the two fractions but the proportion of available sites for incorporation for the unassembled tubulin was only one-half of that for the protein from the polymer. In the absence of exogenous porcine ligase the level of incorporation of ^{14}C-tyrosine in the microtubular fraction was negligible (not shown) indicating that no ligase was associated with pelleted microtubules. The results of this experiment indicated a complementary relationship between the amount of endogenous tyrosine at the

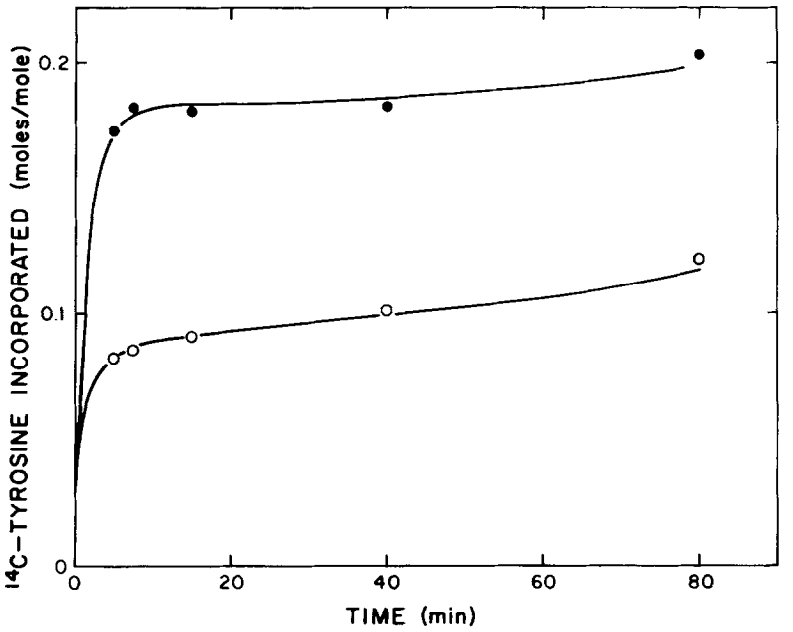

Figure 2. In vitro incorporation of ^{14}C-tyrosine into tubulin of the monomer (o) and polymer (•) fractions from 7-day-old rat brain. Conditions were as described in Materials and Methods. Tubulin content in each fraction was estimated by electrophoresis on SDS-acrylamide gels.

COOH-terminal position and the number of sites available for in vitro incorporation of the amino acid. It also confirms the difference in the state of tyrosination between tubulin of the two fractions from the brains of young animals.

The data shown in this report demonstrate that, at least in early stages of development, tubulin does not distribute at random between the two states of assembly but a selection occurs in the cell which is correlated with the extent of tyrosination of the COOH-terminal end of the α subunit.

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