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MODIFICATION OF THE C-TERMINUS OF BRAIN TUBULIN DURING DEVELOPMENT

Julio A. Rodriguez and Gary G. Borisy

Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53706

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SUMMARY: Tyrosine, as well as small amounts of phenylalanine, were removed selectively and quantitatively from purified chick brain tubulin by enzymatic digestion with carboxypeptidase. The fraction of molecules containing hydrolyzable tyrosine changed with the stage of development and had the highest value ($\sim$0.5) around days 14-16 of the embryo. The increase in the fraction of tyrosinated molecules was found to be temporally correlated with an increased specific activity of the enzyme catalyzing the incorporation reaction. In addition, it was found that the availability of α-chain C-termini for *in vitro* tyrosination also reached a maximum during the same period. Changes in the extent of modification of the C-terminus of tubulin may be relevant for the participation of the resultant microtubules in different developmental events.

Tubulin is the specific substrate for a unique reaction, the post-translational incorporation of aromatic amino acids (tyrosine, phenylalanine or dihydroxyphenylalanine) into the C-terminus of the α-tubulin peptide chain (1-3). Despite the extensive characterization of the reaction (4-7) its biological role remains unknown. It has been reported that tubulin can assemble *in vitro* equally well with or without C-terminal tyrosine (8). This suggests that tyrosination may not be involved in the control of microtubule assembly *in vivo* though this role cannot yet be ruled out. It is also possible that this type of post-translational modification is important in modulating the function of the microtubules *in vivo*. We have studied the reaction as a function of the stage of development of the chick brain in the hope of detecting a correlation with developmental events in the brain. We have examined the state of the C-terminus by two approaches, direct determination of the C-terminal amino acid and determination of the extent to which exogenous tyrosine can be incorporated into the C-terminus. The results of these studies have revealed the existence of a period of maximal modification of tubulin, which we also found to correspond to the period of maximal ligase activity.

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Materials and Methods

Materials: Fertile eggs incubated for different lengths of time at 38° and chickens of different ages were provided by the Poultry Research Lab of the University of Wisconsin-Madison. L-[U-^{14}C]-Tyrosine (486 mCi/mmol) was obtained from Amersham/Searle Co. DEAE-Sephadex A-50, Sephadex G-25, L-tyrosine and 1-nitroso-2-naphthol were from Sigma Chemical Co. Carboxypeptidase A (E.C. 3.4.2.1) treated with diisopropylphosphofluoridate was from Worthington Biochemical Corporation.

Preparation of extracts and protein purification: Extracts from fresh brain tissue were prepared according to the procedure described by Borisy et al. (9) with slight modifications. For the purification of tubulin from high speed supernatants (100,000xg for 60 min) a modification of the ion exchange method of Murphy and Borisy (10) was used. Aliquots of supernatant containing 0.25 M KCl were loaded onto small columns of DEAE-Sephadex A-50 equilibrated with the same saline buffer. For the elution of the tubulin fraction the salt concentration was increased to 0.5 M. Samples were used after desalting by gel filtration on Sephadex G-25 (coarse).

Enzymatic digestion of tubulin: In a typical experiment 5 mg of protein of the desalted tubulin fraction was incubated at 25° for 10 min with 100 µg of prewashed carboxypeptidase in a final volume of 1 ml. The reaction was stopped by addition of perchloric acid, final concentration 0.3 M. The mixture was centrifuged at 10,000xg for 15 min, the supernatant separated, neutralized with KOH and the insoluble $KClO_4$ formed was eliminated by centrifugation. A sample of this supernatant was used for determination of tyrosine by fluorometry. In the controls carboxypeptidase and tubulin were incubated separately and combined after the inactivation step.

Amino acid analysis: The neutralized perchloric acid supernatant after the enzymatic digestion was transferred to a flask of a rotatory evaporator and vacuum dried. The residue was solubilized in a minimum volume of the analyzer buffer and a sample was monitored in a Durrum Model D 500 amino acid analyzer after clarification by centrifugation. A known amount of norleucine was added as internal standard to each sample.

Determination of tyrosine by fluorometry: Tyrosine in the digested material was converted into a fluorescent derivative by reaction with 1-nitroso-2-naphthol according to Waalkes and Udenfriend (11). The fluorescence was measured in an Aminco-Bowman spectrophotofluorometer (slit setting N° 3) equipped with an Aminco Photon Counter attachment. Excitation and emmision wavelengths were set at 460 and 570 nm, respectively.

Determination of ligase activity and capacity of tubulin for amino acid incorporation: The activity of the specific ligase enzyme was measured using the conditions described by Barra et al. (4). For the determination of the maximal acceptor capacity of tubulin for aromatic amino acids the same assay components were used. The acceptor capacity was determined from the plateau level of incorporation in long term incubations (approx. 2 h). Results were expressed in terms of moles of tyrosine incorporated per mol of dimer (M.W. 110,000) after obtaining an estimate of the amount of tubulin in the extract by means of quantitative gel electrophoresis.

Polyacrylamide gel electrophoresis: Samples and gels were prepared and run according to Shapiro et al. (12). Staining with Coomassie brilliant blue was as described by Fairbanks et al. (13). Determination of the degree of purity of tubulin fractions and the estimation of the content of tubulin of the desalted extracts were performed as described by Murphy et al. (14).

Protein concentrations were determined by the method of Lowry et al. (15) as modified by Schacterle and Pollack (16) with bovine serum albumin as standard.

Results and Discussion

Tubulin isolated from the extracts obtained from developing chick brain

as described under Materials and Methods was found to be 90-95% pure as determined by gel electrophoresis. The remaining 5-10% was distributed into several minor bands.

The C-terminal amino acid of native tubulin was determined after digestion with carboxypeptidase. Several studies (7,8,17) have suggested that this enzyme can selectively release the terminal aromatic residues of α-tubulin. The extent of release of C-terminal tyrosine from both in vitro charged tubulinyl-[^{14}C]-tyrosine and native tubulin which had been purified by ion exchange was monitored in preliminary experiments. Results are shown in Fig. 1. Complete hydrolysis of the in vitro bound [^{14}C]-tyrosine was obtained in less than 15 min. A similar kinetic pattern was observed for the release of tyrosine from native tubulin using a fluorometric assay for tyrosine. The inset shows the extent of hydrolysis of tyrosine obtained with different amounts of carboxypeptidase. About 3 μg of enzyme per nmole of tubulin was necessary for complete hydrolysis of tyrosine.

Figure 2 shows the result of an analysis of the acid-soluble material after carboxypeptidase digestion of DEAE-tubulin obtained from 5-week-old chick brain. Tyrosine and phenylalanine were the only amino acids present in the hydrolysate (A) in amounts significantly higher than that in the control (B). From the areas of the corresponding peaks the amount of tyrosine and phenylalanine were calculated to be, respectively, 0.36 and 0.02 moles per mole of tubulin dimer. The amount of phenylalanine found in the 5-week-old chick brain tubulin is in fairly good agreement with that obtained from theoretical calculations (18) based on the Michaelis constants for the incorporation in vitro and the concentration of tyrosine and phenylalanine in brain tissue.

The quantitation of tyrosine was performed in hydrolysates prepared from the tubulin purified at various stages of development by measuring the fluorescence of a nitroso-naphthol derivative of the amino acid (11). It can be seen in Fig. 3 (top) that the fraction of molecules containing hydrolysable

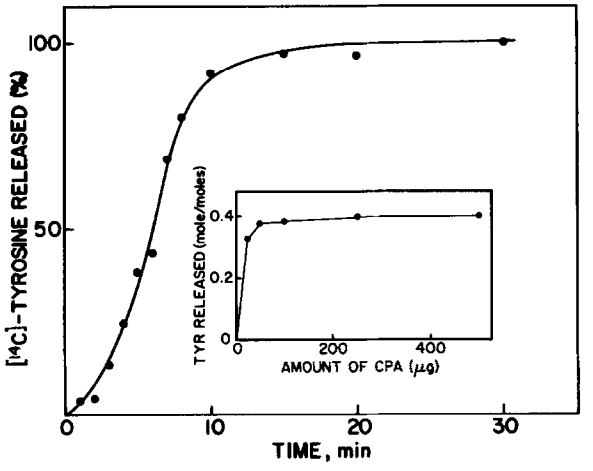

Figure 1. Kinetics of tyrosine release from tubulinyl-[^{14}C]-tyrosine by digestion with carboxypeptidase A. The radioactive complex was prepared as described elsewhere (1). Purification and carboxypeptidase A digestion were according to the details given in the text. Soluble radioactivity after trichloroacetic acid precipitation was measured at the indicated times. Inset: extent of release as a function of the amount of carboxypeptidase used. Thirty nmoles of DEAE-tubulin were digested with the indicated amounts of carboxypeptidase A for 10 min. Tyrosine was measured by the fluorometric technique as described under Materials and Methods.

tyrosine changed with the progress of development. The highest levels of endogenous tyrosine were found during the early embryonic period (days 9 to 16 from the start of incubation of the eggs) in which 44 to 51% of the molecules as isolated from the tissue contained the amino acid. Values began to decrease prior to hatching (day 21) and the percentage dropped to 30% in the adult stage. Also the specific activity of the enzyme (ligase) catalyzing the tyrosination reaction was measured in preparations of developing chick brain. It was found that the neuronal tissue from the earliest stages of embryonic development had significant levels of ligase specific activity. The values increased and rose to a peak around the days 12-16 of the embryo. The enzyme activity then decreased steadily and the adult stage showed very low but still significant levels of enzymatic activity (Fig. 3, bottom). Mixing experiments demonstrated that there was no inhibitor or activator in the brain preparations at any stage (result not shown). A similar time-course for the brain ligase in the early development of the chick was recently reported by Deanin et al. (19).

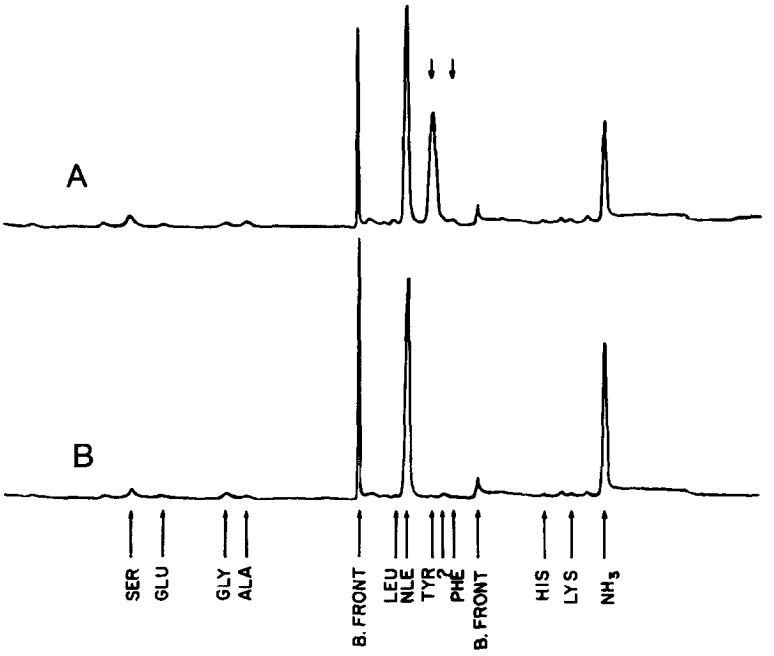

Figure 2. Amino acid analyzer chromatograms of the acid soluble material from DEAE-tubulin incubated in the presence (A) and in the absence (B) of carboxypeptidase A. Details are given in the text. Norleucine (NLE) was added as an internal standard. B. FRONT indicates the peak of material emerging from the column after each buffer change. In some preparations a small peak of an unidentified ninhydrin-positive material was eluted in between the peaks of tyrosine and phenylalanine. Small arrows indicate significant differences between both chromatograms.

While our results indicate tyrosination to be significant throughout development they also indicate that a large proportion of the soluble tubulin molecules were devoid of C-terminal aromatic amino acids at every stage of development analyzed. To confirm this the capacity of tubulin from the chick brain preparations to serve as substrate for amino acylation *in vitro* was determined. Figure 3 (middle) shows the developmental changes of the maximal binding capacity of tubulin measured in extracts with tyrosine as substrate. Availability of sites for further tyrosination *in vitro* was very low during the earliest stages (5 days) in which less than 5% of the molecules were able to accept tyrosine. Then, a rapid increase was observed during the next few days of the embryo and a peak was detected around day 14 of the embryo. At this stage 27% of the molecules had acceptor capacity. A significant decrease

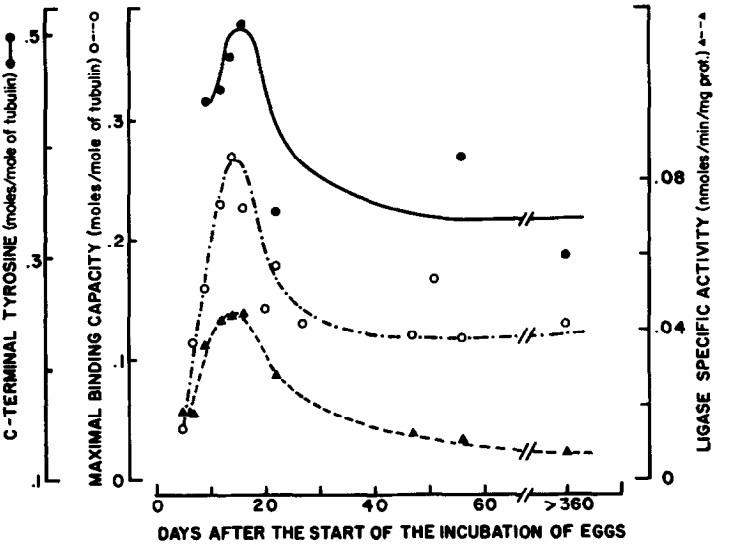

Figure 3. The amount of tyrosine released by carboxypeptidase A digestion from partially purified tubulin, as determined by fluorometry (top, ●—●); the maximal tyrosine-binding capacity of tubulin of the brain extract (middle, o-o), and the specific activity of the enzyme catalyzing the incorporation reaction (bottom, ▲--▲) are plotted as a function of the stage of development of the chicken. In the first case (upper curve), values correspond to duplicate assays of one or two separate experiments with an average deviation less than 10 percent. Values in middle and lower curves represent the mean of at least three separate experiments.

followed, and shortly after hatching a rather constant value was obtained. Thus, the observation of a fractional level of the C-terminal tyrosine content throughout development is supported by these results.

It should also be noted that a portion of the population of tubulin molecules apparently neither contained nor accepted tyrosine, and presumably phenylalanine as well. Tubulin inactivation during the long-term incubation of the extracts can be excluded as a cause for the apparent limit to the capacity of tubulin to accept amino acid, since purified tubulin incubated with partially purified enzyme could be tyrosinated in relatively short time to the same extent as in the two-hour incubations (8). Furthermore, assay conditions for the determination of tyrosine incorporation were those in which release or exchange of C-terminal amino acid were negligible (6,20). Therefore, we suggest that tubulin might undergo some conformational change

which could account for the presence of a form lacking the capability for modification. This possibility is strengthened by the fact that the C-terminal end of α-tubulin appears to have an easy accessibility to both ligase and carboxypeptidase (3,5,8).

The observations described in this paper indicate that soluble tubulin in the chick brain is not a homogeneous population of molecules but consists of at least three forms distinguished by the state of the C-terminal end. The relative proportion of these forms changed during brain development and there was a period during which (a) the content of C-terminal aromatic residue in tubulin, (b) the ability of tubulin to serve as acceptor for tyrosine, and (c) the activity of the specific amino acylating enzyme were all significantly increased. This period around the end of the second week of the embryo is coincident with the one at which tubulin concentration was reported to be maximal in the chick brain (21) and also with that of maximal rate of neuronal growth and synaptogenesis (22). While the details of involvement of tubulin in these events are not known, the presence of intact microtubules has been shown to be required for neurite elongation. Our results, therefore, point to a possible role of tyrosination in regulating the outgrowth of the neuron. Though it has been suggested that tyrosination does not affect the capacity of tubulin to become incorporated into microtubules in vitro (8), it is not known, for example, what controls microtubule elongation or what modulates the associative properties for particular functions of microtubules such as the interaction with other elements within the axonal cytoskeleton.

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