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INTERACTION OF TYROSINE HYDROXYLASE WITH TUBULIN

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SUMMARY : Bovine adrenal medulla tyrosine hydroxylase associates with microtubules during tubulin assembly. Limited proteolytic digestion of tyrosine hydroxylase does not affect the enzymatic activity but prevents its association with tubulin. A possible interpretation is that an ionic interaction occurs between microtubules and a negatively charged region of the enzyme which is removed by the protease treatment. Tyrosine hydroxylase is able to induce purified tubulin assembly as do the microtubule associated proteins ; however, the association induced by tyrosine hydroxylase corresponds to the formation of aggregates or organized structures different from microtubules. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and electron microscopy of proteins obtained from bovine adrenal medulla show the presence of tubulin in this tissue.

Tyrosine hydroxylase catalyzes the rate-limiting step in the biosynthesis of catecholamines in central and peripheral nervous systems and in the chromaffin cells of the adrenal medulla. Some reports on the immunohistochemical localization of tyrosine hydroxylase have suggested that brain tyrosine hydroxylase is associated with a structure which might be neurotubules of dopaminergic and noradrenergic axons (1,2).

Katz et al. (3) have shown that activation of tyrosine hydroxylase by polyelectrolytes such as phosphatidyl-L-serine is the result of an electrostatic interaction between the protein and polyanions. Microtubule associated proteins (MAP) are generally polycationic proteins and some enzymes such as nucleoside diphosphate kinase (4) and tubulin-tyrosine-ligase (5) associate with microtubules. If there is an association of tyrosine hydroxylase with

Abbreviations : MES, 2-(N-morpholino)ethane sulfonic acid ; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid ; MAP, microtubule associated proteins ; RB, reassembly buffer ; GTP, guanosine-5'-triphosphate ; $DMPH_4$, 6,7-dimethyltetrahydropterin ; SDS, sodium dodecyl sulfate ; Tris, Tris(hydroxymethyl)aminomethane.

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neurotubules it could quite reasonably be of an electrostatic nature. To further investigate this possibility tyrosine hydroxylase activity was measured at different stages of tubulin preparations from pig brain and bovine adrenal medulla. In addition, the possible association of the two species, prepared independently has been investigated.

MATERIALS AND METHODS : L-[3,5 ^{3}H]tyrosine, 40 mCi/mmole, was obtained from Amersham and further purified according to Ikeda et al. (6). Poly-L-lysine (molecular weight 2000) was purchased from Sigma and $DMPH_4$ from Aldrich. Catalase was obtained from Boehringer.

Microtubule proteins were isolated from pig brain by three cycles of assembly-disassembly (7). The third cycle was always done just before use. Reassembly buffer (RB) was MES buffer 0.1 M, pH 6.6 containing EGTA 1 mM and $MgCl_2$ 0.5 mM plus GTP where specified. Where stated, tubulin was further purified by chromatography on a phosphocellulose column (8). MAP free of tubulin were prepared by a thermal treatment at high ionic strength (9).

Native microtubules were isolated according to the method of Mann et al. (10) modified by Maury et al.(to be published). The tissue was homogenized in 2 to 3 volumes of buffer -0.1M MES, pH 6.6, 50 % glycerol - centrifuged at 12,000 g for 15 min to remove large debris. The supernatant was then centrifuged at 100,000 g for 45 min. The resulting pellet of native microtubules was resuspended in the homogeneization buffer.

Tubulin protein concentration was determined by the procedure of Lowry et al. using bovine serum albumin as standard (11). The protein composition of these preparations was analyzed by SDS polyacrylamide gel electrophoresis using 5 % - 11 % gradient slab gels (12).

Polymerization and depolymerization were induced by temperature jumps from 5°C to 35°C and from 35°C to 5°C, respectively. The polymers present in solution were identified by electron microscopy after negative staining.

The tyrosine hydroxylase assay was a modification of that described by Nagatsu et al.(13). The standard incubation mixture contained : MES buffer 0.1 M pH 6.2, 2-mercaptoethanol 20 mM, $DMPH_4$ 1 mM, catalase 2000 units, L-[3,5^{3}H]tyrosine 0.1 mM (containing 9×10^5 cpm per assay) and tyrosine hydroxylase in a final volume of 0.4 ml. Incubation was for 15 min at 37°C. Units of tyrosine hydroxylase activity were expressed in nmol of dopa formed per minute. In order to remove catecholamines, all tubulin samples were dialyzed against Tris-HCl buffer,50 mM,pH 7.4 before assaying for tyrosine hydroxylase activity and measuring protein concentrations.

Native tyrosine hydroxylase from bovine adrenal medulla was obtained by filtration on a Sepharose 6 B column (14). The proteolyzed enzyme was prepared by tryptic digestion followed by ammonium sulfate fractionation, separation on DEAE-Sephadex and G-100 gel columns. The detailed purification will be published elsewhere. The enzyme has been estimated to be more than 80 % pure by disc gel electrophoresis.

RESULTS AND DISCUSSION : To investigate the possibility of an in vivo association between tyrosine hydroxylase and tubulin in bovine adrenal medulla, native tubulin prepared from this tissue was first examined. SDS gel electrophoresis showed a doublet migrating as the αβ doublet of tubulin

and electron microscopy showed intact microtubules. The presence of microtubules in adrenal medulla has been previously suggested by the inhibitory action of the antimitotic drugs on exocytosis (15,16). When tyrosine hydroxylase activity was assayed during this preparation about 5 to 10 % of the total activity in the low speed supernatant was found in the pellet of native microtubules and 90 to 95 % in the supernatant, thus showing association of significant amounts of tyrosine hydroxylase with native microtubules. During this kind of preparation there is no loss in total tyrosine hydroxylase activity.

A similar percentage of the total tyrosine hydroxylase activity was found associated with native microtubules prepared from pig brain.

When microtubule proteins were isolated from pig brain by repeated cycles of polymerization-depolymerization (7), total tyrosine hydroxylase activity decreased during the preparation. After the first cycle, 6 % of the total activity present in supernatant and microtubule pellet was associated with microtubules and this enzymatic activity was removed by the second cycle. The loss of activity associated with microtubules was probably due to the decrease in total activity during the preparation and to the loss of any tyrosine hydroxylase associated with microtubules by dilution during each cycle. MAP remaining associated with microtubules may do so because they stabilize this structure.

With the two methods of tubulin preparation used, tyrosine hydroxylase activity found in the microtubules pellet was higher than expected in the pellet volume for a non-interacting protein. Thus, tyrosine hydroxylase might be associated *in vivo* with microtubules. It is tempting to speculate on the physiological significance of this association if tyrosylated tubulin (17) were a substrate for tyrosine hydroxylase.

To determine whether tyrosine hydroxylase interacts *in vitro* with microtubules, a constant level of tyrosine hydroxylase activity was incubated with varying concentrations of tubulin during a fourth cycle of polymerisation. After centrifugation at room temperature the supernatant and the pellet were

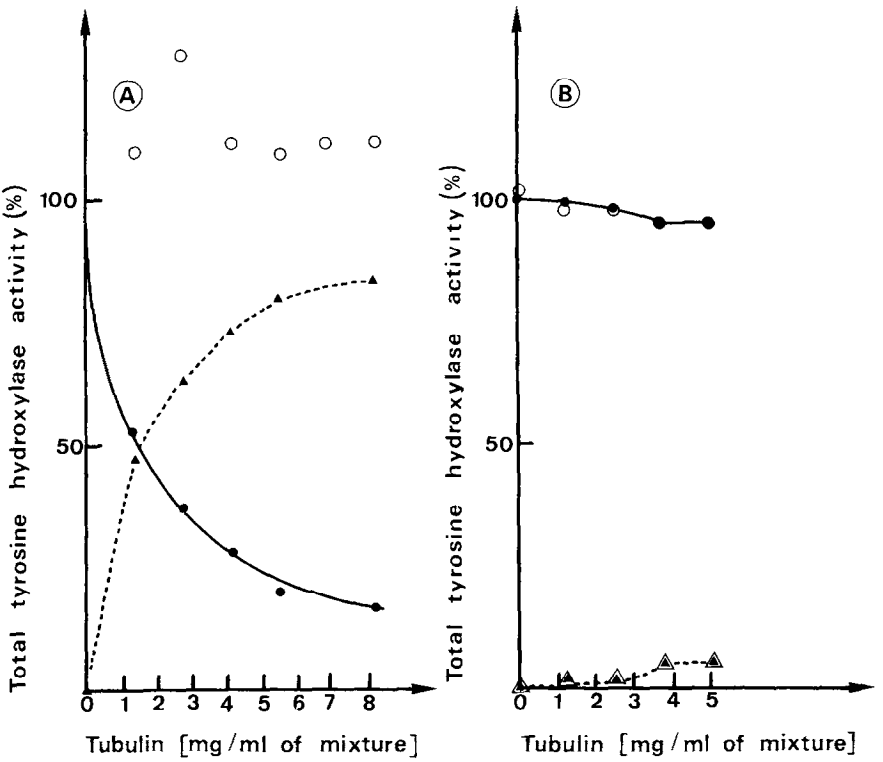

Figure 1-A. In vitro interaction of tyrosine hydroxylase with microtubules. Increasing amounts of tubulin prepared by the Shelanski procedure (7) in RB 1 M glycerol, 0.5 mM GTP were added to constant levels of native form of tyrosine hydroxylase (1.5 units/ml, final concentration) and catalase (1500 units/ml, final concentration) in 50 mM Tris-HCl buffer pH 7. In each case, the final concentration of all components (except tubulin) were maintained constant by addition of RB . Tubulin concentration was always higher than the critical concentration for tubulin self-assembly. After a 30 min incubation at 35°C and centrifugation at 100,000 g at room temperature, supernatants were collected and pellets washed with warm (35°C) RB before solubilization in cold RB 1 M glycerol. Each fraction was tested for tyrosine hydroxylase and catalase activity and its volume measured. For each tubulin concentration added, 100 % of tyrosine hydroxylase or catalase activity is the sum of total enzyme activity in the supernatant (•, tyrosine hydroxylase ; o, catalase) and in the pellet (▲, tyrosine hydroxylase ; △, catalase). Each point represents the mean of duplicate determinations. The experiment was performed three times with similar results.

B. In vitro interaction of proteolyzed tyrosine hydroxylase with microtubules. The same experiment was performed with proteolyzed tyrosine hydroxylase (6 units/ml, final concentration).

assayed for total tyrosine hydroxylase activity. Increasing the tubulin concentration in the incubation mixture decreased the supernatant tyrosine hydroxylase activity and consequently increased the pellet activity (Figure 1-A). A saturation curve for tyrosine hydroxylase activity in the microtubules pellet was obtained indicating a dissociation constant of 15 µM for the microtubule-

Table I. Tyrosine hydroxylase activity associated with microtubules in vitro. Tyrosine hydroxylase (0.75 units), catalase (500 units) and RB 1 M glycerol (320 µl) with or without tubulin (2.4 mg/ml final concentration) in a total volume of 400 µl were heated at 35°C for 30 min. Aliquots of the mixture (150 µl) were centrifuged at 100,000 g for 5 min at room temperature. The supernatant was collected and the pellet washed with warm (35°C) RB 1 M glycerol and homogenized in the same cold buffer at 4°C. The remaining mixture was then incubated for 4 min at 5°C and centrifuged at 35,000 g for 20 min at 5°C. The supernatant was collected and the pellet washed and resuspended as above. In each case 100 % corresponds to the sum of activity present in the supernatant and the pellet.

	+ Tubulin		- Tubulin	
	Total Activity (units)	%	Total Activity (units)	%
35°C (*)				
Supernatant	0.055	<29%	0.23	97.5 %
Pellet	>0.134	>70%	0.006	2.5 %
5°C (**)				
Supernatant	0.23	80%	0.24	100 %
Pellet	0.06	20%	0	0 %

(*) 35°C : Conditions of polymerisation
(**) 5°C : Conditions of depolymerisation

tyrosine hydroxylase complex. Moreover, this saturation curve - in a double reciprocal plot - extrapolates to 100 % bound tyrosine hydroxylase in the pellet. Since there is an equilibrium between dimeric tubulin and polymeric microtubules, this suggests that tyrosine hydroxylase associates more tightly with microtubules than with tubulin. Catalase, which has an isoelectric point (5.8) near that of tyrosine hydroxylase (6.0) has been added to the mixture as a control for non-specific precipitation. It did not associate with the pelleted proteins (Figure 1-A). To verify if tyrosine hydroxylase was associated with microtubules and not with non-specific aggregates, the reversibility of the association was tested. The enzymatic activity in the pellet was measured after an incubation at 35°C (polymerization) followed by cooling at 5°C, conditions under which microtubules depolymerize (Table I). Only 20 % of the activity was associated with the aggregates whereas at 35°C, 70-80 % of the activity was pelleted. In control experiments without tubulin, the enzymatic activity remained in the supernatant. Turbidity measurements have also shown

a very good reversibility of the polymerization in presence of tyrosine hydroxylase. Electron microscopy showed microtubules at 35°C, while after cooling at 5°C, only small aggregates were observed.

Proteolytic digestion did not affect tyrosine hydroxylase activity but after such a treatment the enzyme was no longer activated by phosphatidyl-L-serine (18) suggesting that the enzyme has lost a fragment able to interact with polyanions. To determine if the proteolysis affected the interaction of tyrosine hydroxylase with microtubules, proteolyzed tyrosine hydroxylase was added to various quantities of tubulin proteins during a fourth cycle of polymerization (Figure 1-B). In contrast with the native form, the proteolyzed enzyme did not interact at all with microtubules.

To investigate the electrostatic nature of the interaction, tyrosine hydroxylase was incubated with varying amounts of tubulin during a fourth cycle of polymerization in presence of cations such as Mg^{2+} or poly-L-lysine which might compete with the enzyme for the microtubule association sites. Lee et al. (19) have shown that cations or polycations could substitute for MAP and induce polymerization of tubulin. In our experiements, Mg^{2+} ions did not interfere in the association process (Figure 2), raising the Mg^{2+} concentrations increased only tyrosine hydroxylase inactivation, the curve remaining unchanged. In the absence of tubulin, poly-L-lysine caused the precipitation of tyrosine hydroxylase leaving only 6.5 % in a soluble form. Addition of tubulin to the remaining activity considered to be 100 %, induced the sedimentation of a smaller percentage of the tyrosine hydroxylase activity than that obtained in the absence of poly-L-lysine. This suggests there could be a competition between tyrosine hydroxylase and poly-L-lysine for association with microtubules (Figure 2).

Other results point to an electrostatic interaction between tyrosine hydroxylase and microtubules. The enzyme is adsorbed on heparin or dextran sulfate substituted-Sepharose 6 B and is eluted by salts but is not adsorbed on dextran substituted-Sepharose 6 B. In contrast the proteolyzed enzyme does not interact with any substituted-Sepharose, thus showing the existence of a cationic site

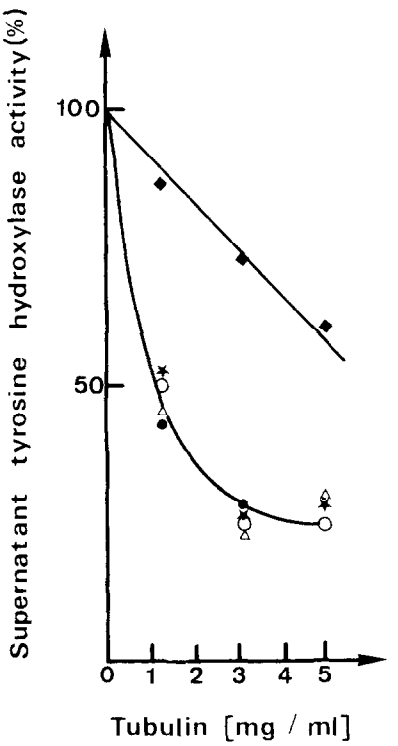

Figure 2. In vitro interaction of tyrosine hydroxylase with microtubules in presence of cations. Increasing amounts of tubulin prepared according to Shelanski (7) in RB 1 M glycerol, 0.5 mM GTP were added to constant levels of native tyrosine hydroxylase (2 units/ml, final concentration) and catalase (1500 units/ml, final concentration) in presence of varying concentrations of Mg^{2+} ions or poly-L-lysine. The final volume was adjusted to 175 µl with RB 1 M glycerol, 0.5 mM GTP. After a 30 min incubation at 35°C and centrifugation at 100,000 g during 5 min at room temperature, the supernatants were collected and assayed for tyrosine hydroxylase activity. The tyrosine hydroxylase activity in absence of tubulin decreased as the concentration of cations was raised ; the tyrosine hydroxylase activity remaining in solution was 92 %, 73 %, 62 % for 0.4 mM, 4 mM, 8 mM Mg^{2+} respectively, and 6.5 % for 8 mM poly-L-lysine monomer. The total enzymatic activity in the supernatant of the mixture without tubulin has been taken as 100 % for each series of measurements at constant cation concentration. Results are represented by (●) 0 mM Mg^{2+}, (+) 0.4 mM Mg^{2+}, (Δ) 4 mM Mg^{2+}, (○) 8 mM Mg^{2+}, (◆) 8 mM poly-L-lysine monomers. Catalase remained in the supernatant.

different from the active site (result to be published). Moreover, the C-terminal sequence of porcine brain α-tubulin is unusually rich in acidic α-amino acid residues (20), supporting the hypothesis of acidic regions in tubulin.

Microtubule associated proteins (MAP) are known to stimulate the initiation and elongation of microtubule assembly. MAP can be separated from tubulin and stimulate microtubule assembly when added back to purified tubulin in RB plus glycerol 1 M, GTP 0.5 mM and $MgCl_2$ 2 mM. Without addition of MAP,

polymerization can occur only in the presence of a higher Mg^{2+} concentration (>5mM). Turbidity measurements and electron microscopy were used to verify these properties for our preparations of MAP and tubulin, and subsequently for tyrosine hydroxylase and tubulin. Addition of tyrosine hydroxylase to tubulin purified on phosphocellulose at low Mg^{2+} concentration induced the formation of insoluble material. Nevertheless, the enzyme did not play the role of MAP, at least for the initiation step of microtubule assembly, since the reaction was not cold reversible and electron microscopy showed aggregates (some of which were ordered in parallel arrays) but not microtubules. The possibility of tyrosine hydroxylase stimulating the elongation step of microtubule assembly was also tested. Tubulin, purified on phosphocellulose, was polymerized in the presence of both MAP and tyrosine hydroxylase. The latter seemed to affect slightly the polymerization kinetics. The rate of assembly was the same with or without tyrosine hydroxylase, but the extent of the turbidity variation was somewhat greater in the presence of tyrosine hydroxylase. Electron microscopy showed the presence of microtubules and aggregates in that case.

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