THE DISTRIBUTION OF TYROSYLTUBULIN LIGASE IN BRAIN AND OTHER TISSUES

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Summary Tyrosyltubulin ligase is widely distributed. It is present in all brain areas, synaptic endings, all of the nonnervous tissues that were examined and in birds as well as mammals. Brain is the only tissue that contains large amounts of endogenous tyrosylatable tubulin. In nervous tissues, ligase activity is not correlated with regions of high catecholamine synthetic capacity.

A ligase is present in rat brain which catalyzes a reversible, post-translational addition of tyrosine (TYR)*, phenylalanine (PA)* and 3,4-dihydroxyphenylalanine (DOPA)* to the carboxyl-terminus of the α-chain of tubulin (1). Its activity is reported to be much greater in brain (50-80 fold) than in kidney, liver and thyroid (2). It seemed possible that the reaction was of special significance for brain function, perhaps one related to catecholamine metabolism.

MATERIALS AND METHODS

MATERIALS: Long Evans rats (18-35 day old males) were purchased from Charles River Laboratories. Fertile White Leghorn eggs were obtained from Spafas, Inc. Aquasol, ^{3}H-L-phenylalanine (1.144 Ci/mmol), ^{3}H-L-tyrosine (0.987 Ci/mmol) and ^{14}C-L-3,4-dihydroxyphenylalanine (0.2315 Ci/mmol) were obtained from New England Nuclear Corporation. ^{14}C- rather than ^{3}H-DOPA was used because of extensive exchange of the tritiated label with water.
METHODS: Tissues were homogenized, 1:1 (w/v), in 0.1 M KCl, 0.05 M Tris-HCl, pH 7.5, 5 mM Mg acetate, 1 mM dithiothreitol (TKMD buffer) and centrifuged for 20 min at 15,000 rpm in a Sorvall refrigerated centrifuge. The supernatant fraction was then centrifuged for 1.5 h at 230,000 x g_{av}, the upper 80% removed and dialyzed against several changes of homogenizing medium containing 10% glycerol. The dialysate which has ligase activity was stored in liquid nitrogen and used after storage for 3-4 weeks.

Rat brain tubulin was prepared and purified by 2 cycles of assembly and disassembly, as described by Shelanski (3), and stored at -60° in reassembly buffer. Tyrosine binding capacities of these preparations remain constant for at least 3 mos.

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*Abbreviations:
TYR, tyrosine; PA, phenylalanine; DOPA, 3,4-dihydroxyphenylalanine.

Ligase activity was measured by incubation in TKMD buffer supplemented with 2.5 mM ATP, sufficient Mg acetate to achieve a concentration of 10-11 mM, 1 M glycerol, radioactive amino acid, purified tubulin and supernatant enzyme fraction as specified in tables and figure legends. Following incubation at 37° for 10 min, samples were taken for evaluation of trichloracetic acid (TCA) precipitable radioactivity on filter paper disks (4) and/or prepared for analysis by SDS polyacrylamide gel electrophoresis (see below).

Synaptic endings were prepared from brains homogenized in 0.32 M sucrose. The crude mitochondrial pellet was isolated and washed as described by Barondes (5). The washed pellet was fractionated on sucrose gradients as described by Gray and Whittaker (6). The synaptic ending layer was collected, diluted to 0.32 M sucrose and centrifuged for 15 min at 15,000 rpm. The pellet was washed 4 X with 0.2 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.32 M sucrose, 2 mM 2-mercaptoethanol (NTSM buffer).

Washed synaptic endings were gently resuspended in NTSM buffer and supplemented with 0.02 mM radioactive amino acid. The suspensions were incubated for 10 min at 30°, diluted with a tenfold volume of cold NTSM buffer and centrifuged for 15 min at 15,000 rpm. The pellets were washed 4 X by repeated centrifugations with NTSM buffer and resuspended in 1 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, 2 mM 2-mercaptoethanol. Samples were assayed for free and TCA precipitable radioactivity (4). Part of the lysed suspension was centrifuged for 1 h at 200,000 x g_{av}; the supernatant fraction containing the soluble synaptic ending proteins and the original total suspension were prepared for SDS gel electrophoresis (see below).

Unincubated, washed synaptic endings were lysed and centrifuged as above. The supernatant fraction was dialyzed overnight against TKMD buffer containing 10% glycerol and ligase activity was measured as described for the whole brain supernatant fraction.

SDS gel electrophoresis was performed on 7.5% gels as described by Laemmli (7); this procedure separates the α and β chains of tubulin (8). Following electrophoresis, gels were stained with Coomassie blue and destained in 7.5% acetic acid: 7.5% methanol. Two millimeter slices were dried at 50°, dissolved in H_2O_2 and counted in Aquasol. Efficiency was measured directly by spiking samples of dissolved gels with ^{14}C- or ^{3}H-toluene.

Protein was measured by the procedure of Lowry *et al.* (9) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

The tyrosylatable tubulin in brain supernatant enzyme preparations is labile. Incubation for 20 min at 37° abolishes 50% of the TYR binding capacity (10). Even on storage in liquid nitrogen, binding capacity is lost within 3-4 weeks, while ligase activity remains largely unchanged. Therefore, evaluation of ligase activity was performed in the presence of sufficient purified brain tubulin (11) to insure nonlimiting amounts of this substrate. Under these conditions, linear binding of TYR occurs for at least 20 min. The lowest k_m estimate reported by Barra *et al.* (12) for TYR, 0.02 mM, was confirmed.

The binding of PA, TYR and DOPA to purified tubulin was measured in sep-

TABLE 1. BINDING OF AROMATIC AMINO ACIDS TO TUBULIN

Amino Acid in Incubation Mixture	Total TCA-Precipitable Radioactivity		Radioactivity Associated With α-Monomer on SDS gels	
	pmols mg total protein	% of Tyrosine	pmols mg total protein	% of Tyrosine
A. WHOLE BRAIN SUPERNATANT FRACTION + EXOGENOUSLY SUPPLIED TUBULIN				
DOPA	17.62	32.82	1.14	2.25
PHENYLALANINE	2.46	4.57	1.84	3.62
TYROSINE	53.69	100.00	50.77	100.00
B. TOTAL SYNAPTIC ENDING PREPARATION CONTAINING INTRASYNAPTOSOMAL TUBULIN				
DOPA	7.33	234.9	0.14	3.96
PHENYLALANINE	0.035	1.12	0.22	6.27
TYROSINE	3.12	100.00	3.51	100.00
C. SUPERNATANT FRACTION FROM LYSED SYNAPTIC ENDINGS CONTAINING SOLUBLE INTRASYNAPTOSOMAL TUBULIN				
DOPA	-	-	0.42	7.74
PHENYLALANINE	-	-	0.62	11.43
TYROSINE	-	-	5.44	100.00

A. - Incubation mixture (see methods) also contains 1.07 mg whole brain supernatant protein, 1.54 mg purified rat brain tubulin, 0.4 mg RNAse and 20 nmols of either ^{14}C-DOPA, ^{3}H-PA or ^{3}H-TYR, in a total volume of 1 ml. Mixtures were incubated for 10 min at 37°. Approximately 0.35 mg of protein was applied to the gel after treatment with SDS (see methods).

B. & C. - Suspensions, containing approximately 10 mg synaptic ending protein/ml (see methods for additional components) were incubated for 10 min at 30° with either 0.02 mM ^{14}C-DOPA, ^{3}H-PA or ^{3}H-TYR. During this time 0.21 nmols ^{14}C-DOPA, 0.14 nmols ^{3}H-PA or 0.28 nmols ^{3}H-TYR were accumulated/mg synaptic ending protein. Following incubation and washing, the endings were lysed and part of the mixture was centrifuged to recover the supernatant fraction which contains soluble synaptic ending proteins. Approximately 1 mg total synaptic ending protein or 0.2 mg of supernatant fraction were applied/gel after treatment with SDS.

arate experiments. At equimolar concentrations, 0.02 mM, the amount of TYR bound to total TCA precipitable protein was 3 X greater than that of DOPA and 30 X greater than that of PA (Table 1). On SDS gels, only a single peak of radioactivity, co-migrating with the α-chain of tubulin, was observed (Fig. 1); the amounts of DOPA and PA bound were approximately equal, 3% that of TYR. Apparently, even in the presence of ascorbic acid (12), there is some

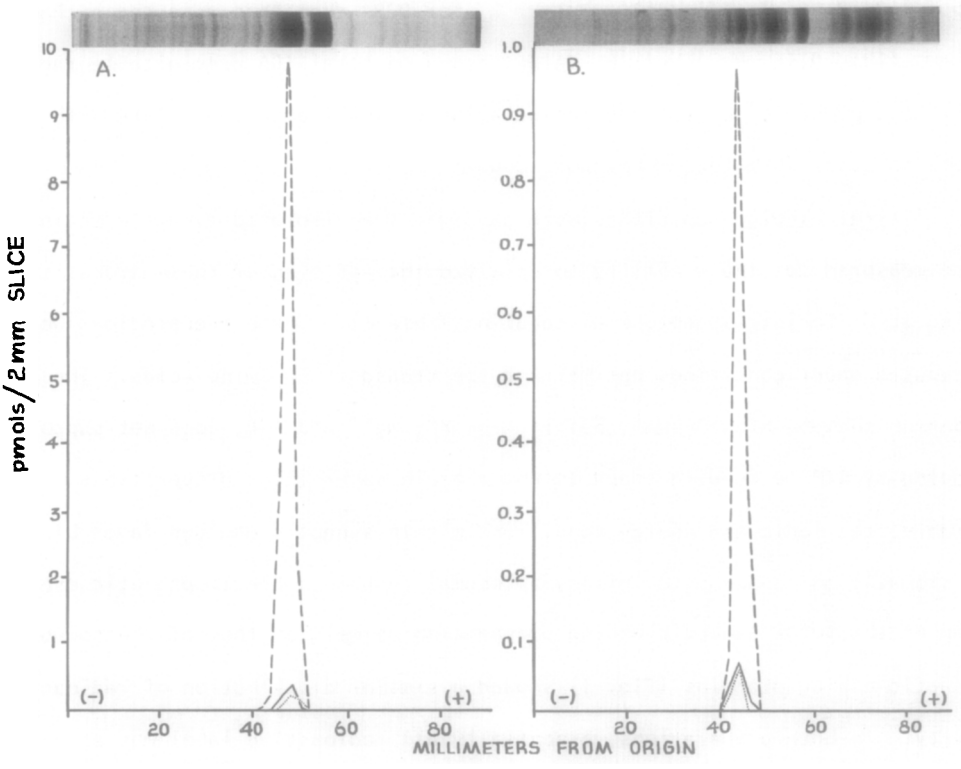

Figure 1: Distribution of radioactivity in SDS-electrophoretic gels. A. Whole brain supernatant fraction + exogenously supplied tubulin. B. Supernatant fraction from lysed synaptic endings containing soluble intrasynaptosomal tubulin. (See Table 1 for details.) DOPA·····, PA———, TYR-----.

association of DOPA with protein, which survives hot TCA treatment, but which is lost on exposure to SDS.

If the same enzyme catalyzes the reaction between tubulin and these three amino acids, a single point estimate of their relative Michaelis-Menton constants can be obtained; for example,

$$k_m\ (PA) = \left[\frac{v(TYR) \cdot S(PA)}{v(PA) \cdot S(TYR)} \cdot [k_m\ (TYR) + S(TYR)] \right] - S(PA)$$

Using 0.02 mM as the k_m for TYR, we obtained an apparent k_m of 0.1 mM for DOPA and 1.2 mM for PA, based on total TCA precipitable radioactivity. Data from SDS gels yielded an apparent k_m for DOPA which approximates that of PA,

about 1.2 mM, a significantly greater k_m for DOPA than that reported by Barra *et al.* (12), 0.16 mM. Their k_m value, based on total TCA precipitable radioactivity, probably reflects the association of small amounts of DOPA with proteins other than the α-chain of tubulin.

Similar binding capacities were observed when isolated synaptic endings were measured for their ability to catalyze the addition of these aromatic amino acids to intrasynaptosomal tubulin (Table 1). These preparations were incubated under conditions optimal for the transport of amino acids. The incubation medium, high in Na^+, but lacking K^+, Mg^{++} and ATP, does not support binding of TYR to tubulin added to whole brain supernatant preparations. Presumably, the ionic and energy conditions within synaptic endings favor binding of aromatic amino acids to intrasynaptosomal tubulin. Electrophoretic evaluation of the total synaptic ending preparation as well as that of the soluble synaptic ending proteins (Fig. 1) showed a similar distribution of radioactivity. In both preparations, essentially all radioactive label was associated with protein co-migrating with the α-tubulin monomer.

Considering the concentrations of the aromatic amino acids in brain (13, 14) and their relative affinities for tubulin, special conditions must be present in intact tissue to permit significant binding of either DOPA or PA. Such conditions are clearly not present in isolated synaptic endings. The internal concentration of radioactive DOPA, TYR and PA in synaptic endings following incubation for 10 min was in the range of 0.05-0.1 mM, equivalent to or many times higher than that found in brain *in vivo* (13, 14). Even under these conditions, binding of DOPA and PA was only 3-5% of TYR.

The ligase activity of the dialyzed supernatant enzyme fraction prepared from washed synaptic endings is similar to that of the whole brain supernatant enzyme fraction (Table 2). Apparently, there is little or no gradient in ligase activity in the neuron. These preparations were also compared with those of whole brain for their ability to hydrolyze rat brain tyrosyltubulin purified after labeling with ^{3}H-TYR *in vitro*. No significant hydrolysis oc-

TABLE 2. TYROSYLTUBULIN LIGASE ACTIVITY IN SUPERNATANT FRACTIONS ISOLATED FROM RAT AND EMBRYONIC CHICK TISSUES

Tissue Source	pmols ^{3}H-Tyrosine mg Supernatant Protein	% of Rat Whole Brain Activity
RAT TISSUES		
Whole Brain	189.5	100.00
Caudate-Striatum	153.5	81.1
Cerebellum	95.4	50.4
Cortex	209.3	110.5
Hypothalamus	92.8	49.0
Hindbrain	157.8	83.3
Synaptic Endings	103.0	54.4
Whole Adrenal	71.6	37.8
Kidney	20.7	10.9
Liver	28.6	15.1
Lung	42.6	22.5
Spleen	42.1	22.2
Testis	25.7	13.5
EMBRYONIC CHICK TISSUES (14 day)		
Whole Brain	260.9	137.7
Muscle (thigh)	406.6	214.5
Liver	58.0	30.6

Reaction mixture and conditions of incubation were as described in legend for Table 1-A with these differences: supernatant protein - 0.5 mg/ml; purified rat brain tubulin - 1.0 mg/ml; ^{3}H-TYR - 25 nmols/ml. Rat and chick supernatant fractions also catalyze the binding of ^{3}H-TYR to purified chick brain tubulin.

curred during incubation at 37° for 60 min in the presence of added Mg^{++}, K^+ and ATP, conditions designed to mimic those of intact synaptic endings except that free TYR was omitted to prejudice the equilibrium toward dissociation. If tyrosyltubulin serves as a storage supply for tyrosine in the neuron, some hydrolytic activity might have been expected under these conditions.

Neither the distribution of tyrosyltubulin ligase activity in the neuron nor the synthesis of tyrosyltubulin in synaptic endings tests the possibility that this reaction is somehow involved in catecholamine metabolism. Tyrosine hydroxylase is found in high concentration in synaptic endings as well as in high speed supernatant fractions from brain (15). Unlike tyrosine hydroxylase, however, ligase activity in brain (Table 2) is not correlated with areas rich in catecholamine synthetic capacity. Ligase activity is fairly uniformly

distributed; the region of highest ligase activity - cortex - has relatively low tyrosine hydroxylase activity (15).

The report of Barra *et al.* (2) that ligase activity is very low in non-nervous tissue was based on the assumption that these tissues, like brain, contain significant concentrations of tyrosylatable tubulin. Little or no ligase activity can be demonstrated, even in fresh preparations from these tissues, in the absence of added tubulin. When ligase activity is evaluated in the presence of exogenously supplied brain tubulin, however, the enzyme is readily demonstrable in all of the rat tissues that were examined (Table 2). No tissue has less than 10% of the activity found in brain/mg supernatant protein. In embryonic chick tissues, during certain periods of development, the ligase activity of the supernatant fraction from thigh muscle exceeds that of brain (Table 2), though the ligase activity of brain is greater than muscle after hatching (16).

The ubiquitous distribution of the ligase, its high activity in embryonic tissues and the compatibility of both substrate and enzyme from mammals and birds are all consistent with some important role in cell function. Whether the localization of measurable endogenous tyrosylatable tubulin to brain implies some special role of this species in the nervous system is not yet clear. Its apparent absence in other tissues may be an artifact of the preparative procedures employed; all of the tubulin in these tissues may already be tyrosylated; or, the degradative phenomena observed with brain fractions may occur at a greater rate in nonnervous tissues. Experiments are now in progress to evaluate these possibilities.

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