AN ENZYME TYROSYLATING α-TUBULIN AND ITS ROLE IN MICROTUBULE ASSEMBLY

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SUMMARY. Alpha tubulin is shown to be the only polypeptide in brain extracts which undergoes C-terminal addition of a tyrosine residue. Tubulin purified by cycles of *in vitro* polymerization can be used as substrate to assay and purify the tyrosine ligase. Tubulin dimer is a substrate for the enzyme and axonemal doublet microtubules are not. Both tyrosylated and untyrosylated dimers can polymerize *in vitro*.

In living cells microtubules may form and dissolve rapidly at times when the total cell content of tubulin appears to be constant. Although all tubulins studied appear to be structurally similar, it is possible in some cases (1) that there may be multiple genes coding tubulins destined for one or another specific organelle. More generally it seems that transcriptional and translational controls could not be sufficient to regulate microtubule assembly *in vivo*. Reversible post-translational modifications of tubulin are therefore intriguing candidates to modulate the assembly, or function, of microtubules. One example is the binding, and certain transformations, of guanine nucleotides. Another modification is the phosphorylation of serine residues in β-tubulin, as well as in several proteins which appear to copolymerize with tubulin; however no evidence has appeared that these phosphorylations can be reversed.

Barra and coworkers (2-4) have described a reaction in which brain extracts incorporate tyrosine as a single unit into the C-terminal position of a protein, apparently through a peptide bond to the α-carboxyl of glutamate (5). The reaction, which appears unprecedented, could not utilize tyrosyl-tRNA (2), and required only ATP. Moreover it was reversible in the presence of ADP + P_i (6). Recently these authors have described a number of similarities between the protein that incorporates tyrosine and tubulin (4).

METHODS. Rat or bovine brains from freshly sacrificed animals were homogenized at 0° with a teflon and glass homogenizer or a Waring blendor. Supernatants from centrifuging one hr at 0° at 100,000 x g were stored at -80°, or used at once to purify tubulin. In the latter case extraction was done in polymerization buffer: 100 mM Na^+MES(2(N-morpholino)ethane sulfonic acid), pH 6.6, 0.5 mM $MgSO_4$, 1 mM EGTA, 1 mM GTP. Unless otherwise specified tubulin was purified by 3 cycles of polymerization and depolymerization as described by Shelanski et al. (7), but without glycerol. In each cycle microtubules are polymerized at 37°, pelleted at 25-30°, the pellets are depolymerized at 0°, the chilled solution again centrifuged for 1 hr at 0°, and the cold pellets discarded. The final warm pellet was dissolved in a small volume of cold polymerization buffer and stored at -80°.

To prepare tyrosine ligase extraction was done in 50 mM K^+MES, pH 6.6, 1 mM mercaptoethanol. The tubulin present in these fresh extracts (see Fig. 2) is denatured in a few days, even at -80° (see Table II). "Enzyme" refers to such aged extracts or to ammonium sulfate or other fractions as indicated.

The colchicine binding capacity of tubulin was assayed as previously described (8). Competence to polymerize was assayed by a small scale centrifugation procedure similar to that used to purify tubulin. From 250 to 500 µg of protein was incubated for 30 min at 37° in 0.25 ml of 100 mM Na^+MES, pH 6.6, 0.1 mM $MgSO_4$, 1 mM GTP, then centrifuged at 25-30° for 20 minutes at 35,000 x g. Polymerizability = % of protein pelleting less % in a companion incubation with 0.1 mM colchicine. Protein was determined by the Lowry procedure (9).

The tyrosylation reaction was measured (2) at 37° in 0.1 ml final volume containing tubulin and enzyme as indicated and: 25 mM Tris.HCl, pH 7.0, 30 mM KCl, 12.5 mM $MgCl_2$, 2.5 mM ATP and, unless otherwise indicated, 0.1 mM (50 µCi/µmole) L-[U-^{14}C]tyrosine (Amersham/Searle), and 0.1 mM colchicine. Incorporation of tyrosine into protein was determined by a modification of the filter paper disc method (10). Results are expressed as nmoles of tyrosine incorporated per nmole of tubulin dimer, or per 110 µg of protein (the equivalent weight). A unit of ligase catalyzes incorporation of 1 nmole in 1 minute when saturated with tubulin.

RESULTS. Fig.1 (A-C) shows that when a fresh brain extract is incubated with [^{14}C]tyrosine and then fractionated by SDS gel electrophoresis, only one protein band is labeled and its mobility corresponds to α-tubulin. Gels were overloaded to permit detection of traces of any other labeled proteins. The same result was obtained with a second electrophoretic system (13). When the labeled extract was fractionated, those fractions enriched in protein-bound tyrosine proved to also be enriched in tubulin (Fig. 1, D-E).

Tyrosine fixation by a fresh extract stops after about 10 minutes (Fig. 2). Tubulin is labile under most conditions, and extracts lose all ability to fix tyrosine on storage. The ability is restored by adding tubulin purified by 3 cycles of polymerization, which by itself (Fig. 2) also does not fix tyrosine (tubulin purified by salt and DEAE fractionation (14) still retains some enzyme activity). Thus the ligase can be separated from the tubulin acceptor substrate. The enzyme is saturated with 2 nmoles (220 µg) of tubulin (Fig. 2) and thus can

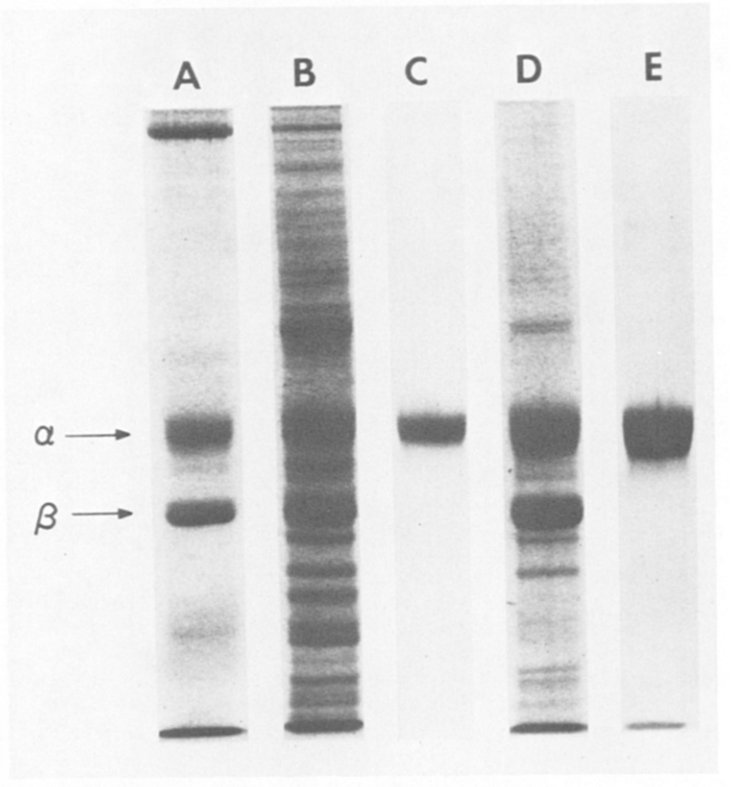

Figure 1. Specific tyrosylation of α-tubulin. A fresh enzyme extract of rat brain (36 mg protein in final volume of 4.5 ml) was incubated for 30 minutes at 37° without further addition of tubulin, under tyrosylating conditions as described in Methods except that the [^{14}C]tyrosine was 0.01 mM and the specific activity 520 μCi/μmole. One aliquot was applied to a Sephadex G-50 column to remove unbound tyrosine. A second was applied to a column of DEAE-cellulose and eluted with a gradient of 0 to 0.8 M KCl in 10 mM Tris.HCl, pH 7.2, and 5 mM mercaptoethanol. Fractions containing acid insoluble radioactivity were pooled. The 2 aliquots were reduced and carboxymethylated in 8 M urea and analyzed on SDS polyacrylamide slab gels in the Luduena and Woodward (11) modification of the Davis procedure (12). The origin is at the top. A is reference bovine brain tubulin after 3 cycles of polymerization, B the aliquot passed through Sephadex, and D the fraction from DEAE-cellulose, stained with Coomassie Brilliant Blue. C and E are autoradiograms of the gels in B and D, respectively.

be assayed even in fresh extracts: The specific activity is 0.035 units/mg in rat or bovine brain extracts. Rates are the same if reaction mixtures contain 0.1 mM colchicine or 1.0 mM Ca^{+2} to prevent tubulin polymerization (but 10 mM Ca^{+2} inhibits).

The enzyme is stable for a month at -80°, is variably inactivated by dialy-

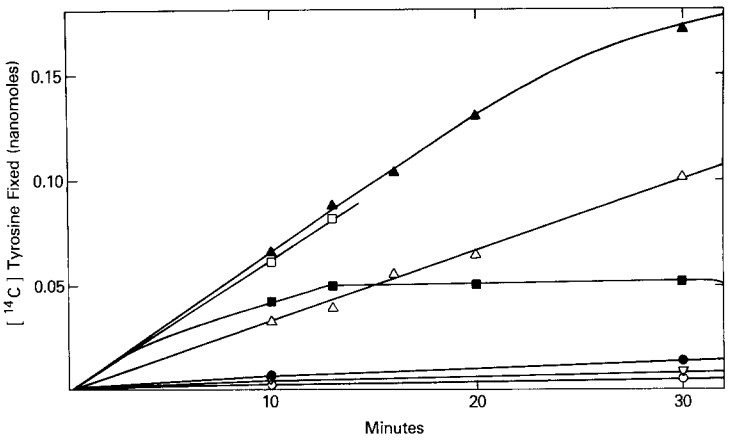

Figure 2. Assay of tyrosine ligase from bovine brain. Fresh enzyme extract without additional tubulin, ~0.006 enzyme unit (■); enzyme (aged extract), 0.006 unit (▽); tubulin, 2 nmoles (○); enzyme 0.003 unit, tubulin 2 nmoles (△); enzyme 0.006 unit, tubulin 2 nmoles (▲); enzyme 0.006 unit, tubulin 4 nmoles (□); enzyme 0.006 unit, tubulin 2 nmoles, incubated at 16° (●).

sis, and is more heat sensitive than tubulin: 87% denatured in 2 minutes at 52° (22% for tubulin). It has not yet been much purified (5 fold). On filtration through Sephadex G-200 it appeared monodisperse with an approximate molecular weight of 150,000. However in the presence of particulate material it seems to be adsorbed and the reaction inhibited, which may be the reason for failure to fractionate sharply in several procedures; recombination of fractions has not indicated the existence of more than 1 component. We confirmed the cofactor requirements for the reaction (2); GTP can replace ATP but is less effective. Aged extract can also liberate free tyrosine from labeled tubulin prepared as in Table I, in the presence of ADP + P_i (6).

Which molecular species of tubulin are substrates for the ligase? Relevant species are α-monomer, 6s dimer, 36s rings and microtubule polymer. It seems unlikely that tyrosylation is required for monomer → dimer or that α-monomer is a substrate; it is not known to exist in equilibrium with other species. Cold depolymerizes microtubules to a mixture of 6s and 36s (Ca^{+2} does also; colchicine can yield some >50s ribbons of protofilaments (15)). When these species were se-

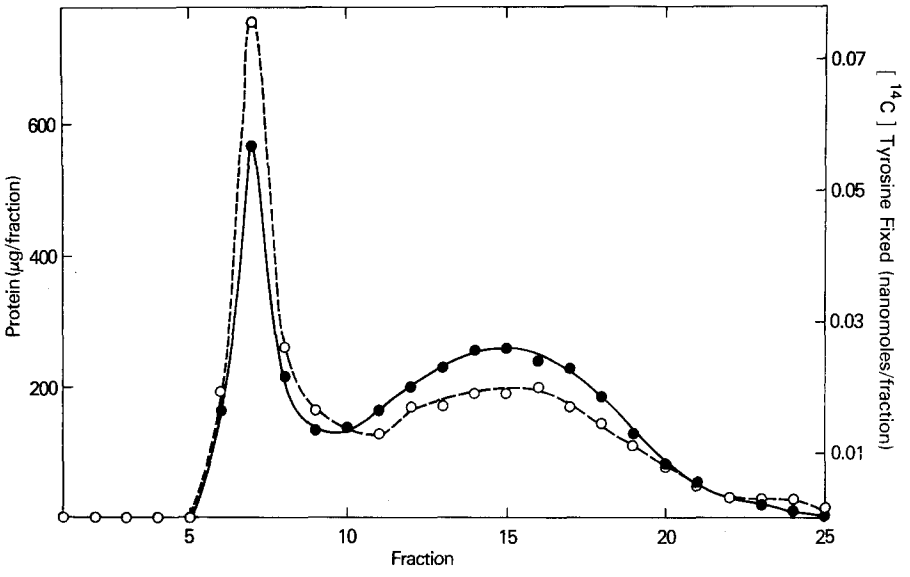

Figure 3. Partition of ^{14}C between 6s (fractions 10 to 20) and 36s (fractions 6 to 9) species of tubulin after depolymerization of tyrosylated microtubules. [^{14}C]Tyrosylated tubulin was purified by 4 cycles of polymerization. An aliquot (0.3 ml, 3.2 mg) depolymerized by chilling was applied to a 15 ml bed of 4% agarose 15m, 50-100 mesh. The column was equilibrated and eluted (1 ml/fraction) at 2° with polymerization buffer (0.2 mM GTP). Tyrosine fixed (—●—), protein (--O--).

parated on an agarose column as in Fig. 3, they had equivalent capacity (see Table II) to accept tyrosine (note the effect on these values for the parental tubulin of varying tubulin and enzyme concentration). This 6s dimer cannot form 36s or microtubules (15), and we conclude that it is a substrate for the ligase. We cannot conclude the same for 36s since it may be in equilibrium with 6s. Fig. 3 shows the result of applying [^{14}C]tyrosylated tubulin to the agarose column: ^{14}C appears enriched in the 6s fraction, but this is due to the fact that 12% of the total protein is not tubulin. We find that this is the % of copolymerizing protein and/or impurities after 3 cycles, and that all of these are in the 36s fraction. Therefore when tyrosylated tubulin is depolymerized by cold, tyrosylated species partition indiscriminately between 6s and 36s.

An aliquot of 3 cycle purified tubulin was repolymerized for 30 min at 37°

TABLE I

Polymerizability of Tyrosylated Tubulin

Tyrosylation was for 40 minutes at 37° in 5 ml containing 35 mg of 3-cycle purified tubulin, 0.9 unit (25 mg) of enzyme fraction and 0.5 μmole (25 μCi) of [^{14}C]tyrosine. The reaction mixture contained 50 mM MES buffer, pH 6.6 (instead of Tris) and, in addition to the usual components, 1 mM $CaCl_2$, 0.5 mM mercaptoethanol, and 0.3 mM GTP. After chilling and adding 12 μmoles of EGTA, free tyrosine was removed at once by passing the mixture through a column of Sephadex G-50 equilibrated with polymerization buffer at +2°. The protein fraction was then subjected to repeated cycles of polymerization.

Fraction	Protein (mg)	Colchicine bound (nmoles)	[^{14}C]Tyrosine Total nmoles fixed	[^{14}C]Tyrosine Free (% of total)	Tubulin (nmoles)[a]	Moles [^{14}C]tyrosine fixed per mole tubulin: Microtubule fraction	Moles [^{14}C]tyrosine fixed per mole tubulin: Non-polymerizing fraction
Sephadex eluate	52	36	14.3	0	180	(0.079)	
1st Warm supernatant	23	12.7	3.5	6.8	64		0.055
1st Cold supernatant	18	16.8	4.6	0	84	0.055	
1st Cold pellet	4.0	3.2	0.64	0	16		
2nd Warm supernatant	4.1	4.4	1.5	3.8	22		0.068
2nd Cold supernatant	13	14	5.4	0	80	0.068	
2nd Cold pellet	0.7	1.1	0.3	0	6		
3rd Warm supernatant	1.3	2.0	0.58	2.5	10		0.058
3rd Cold supernatant	11	20	4.21	0	100	0.042	
3rd Cold pellet	0						

[a]The amount of native tubulin was calculated by assuming that 110 μg (one nmole of dimer) bound 0.20 nmoles of colchicine.

at a concentration (20 mg/ml) at which the solution set to a gel. On 5-fold dilution into tyrosylation mixture it was tyrosylated at the same rate (10 min incubation) and to the same extent (120 min) as a companion aliquot kept at 0° before the reaction. Microtubules might depolymerize quickly in tyrosylating mixture due to the high Mg^{+2}. We therefore tested axonemal outer doublet microtubules, which do not depolymerize in these conditions. No tyrosine acceptor, or ligase activity, was detected (the assay allowed detection of 10 and 5%, respectively, of the levels in brain extract), when the following were supplemented with brain enzyme or tubulin: Tetrahymena cilia demembranated with 0.5% Nonidet P40, isolated axonemes, extract containing central pair tubulin, and outer doublet

TABLE II

Minimal Capacity of Different Tubulin Preparations to Accept [^{14}C]Tyrosine

The extent to which tubulin could be tyrosylated was estimated by using 2 hr incubations with relatively little tubulin and large amounts of enzyme (except in the experiment of the bottom 3 lines). In the former case no more tyrosine was fixed when more enzyme was added and the incubation continued another hr.

Tubulin preparation	Percent of protein competent to polymerize	Colchicine binding (nmoles per 110 µg)	Addition to reaction mixture: Enzyme (unit)	Addition to reaction mixture: Tubulin (nmoles)	Tyrosine fixed (nmoles/nmole of tubulin)[a]
Brain extract (enzyme)	0	0.0004	0.019	-	0
Brain extract (tubulin)	10	0.025 ± .005	0.019	-	0.032 ± .015
2 Cycles, 6 months old	0	0.22	0.018	0.5	0.23
3 Cycles, 6 weeks old	55	0.24	0.018	0.5	0.26
3 Cycles, 10 days old	70	0.25	0.018	0.5	0.31
"	70	0.25	0.0063	3.5	0.13
" , 6s fraction[b]	0		0.0063	0.13	0.25
" , 36s fraction[b]	(+)		0.0063	0.62	0.18

[a]Or per 110 µg of protein in tubulin preparation.

[b]Peak fractions from an agarose 15m column as in Fig. 3.

microtubules (16). All particulate fractions inhibited the reaction observed with both brain components.

How does addition of tyrosine affect the competence of tubulin to polymerize in vitro? When 3 cycle purified tubulin was tyrosylated and polymerized 3 more times, there was no significant enrichment of ^{14}C in tubulin which did or did not polymerize (Table I). Thus C-terminal tyrosine does not block incorporation of tubulin into polymer. Decreasing amounts of free tyrosine were released during the warm incubations of successive polymerization cycles (Table I), showing that the tyrosine removing activity, as well as the ligase, was being fractionated away. Work is in progress to determine whether the 2 activities reside in the same enzyme.

Pending direct C-terminal analyses, we tried to estimate the proportion of brain tubulin which is tyrosylated by determining its capacity to accept additional [^{14}C]tyrosine in long incubations with large amounts of enzyme. Values are mini-

mal since tubulin is denatured during the reaction: 1/3 of the colchicine binding activity was lost in the 40 min incubation of Table I. Table II shows that tyrosine acceptor capacity parallels colchicine binding. At least 30% of the tubulin appeared to lack C-terminal tyrosine. Since 70 to 90% (Tables I and II) of this tubulin polymerizes, we can conclude that tubulin without C-terminal tyrosine can also be incorporated into polymer.

Both tyrosylated and detyrosylated tubulin seem able to polymerize in vitro. It is possible that some of each is required, or that the requirements are more stringent in vivo. More purified enzyme will help answer the question. The enzyme is specific for tyrosine among other amino acids (3) but the true substrate could be something else (i.e. a membrane protein with N-terminal tyrosine). We do not know for certain that cellular tubulin is tyrosylated at all, and hope to determine this by physically separating the 2 species of α-tubulin. Tyrosylation is not the basis for the resolution of bovine α-tubulin into 2 components on SDS hydroxylapatite columns (17), since we find that the radioactivity of tyrosylated tubulin partitions indiscriminately between the 2 components.

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