Enzyme Which Specifically Adds Tyrosine to the α Chain of Tubulin†

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ABSTRACT: A posttranslational modification of tubulin with potential significance for the regulation of microtubule assembly or function has been revealed by the discovery of an enzyme (tubulin–tyrosine ligase) which can add a tyrosine residue to the α chain, apparently through peptide bond linkage to a C-terminal glutamate or glutamine. The ability to add tyrosine in the presence of ATP and to release it again in the presence of ADP and inorganic phosphate (or arsenate) appear to be functions of the same enzyme, as judged by the relative rates at which these reactions are catalyzed over a 20-fold enzyme purification. The apparent size of the enzyme from bovine brain is 150 000 daltons in extracts and after ammonium sulfate fractionation, but 35 000 after elution from anion-exchange columns. Addition of pure dimeric tubulin to the latter species converts it back to the larger form, which is apparently a stoichiometric 1:1 complex of tubulin and the 35 000-dalton enzyme. Tubulin–tyrosine ligase is specific for tubulin; other proteins with C-terminal glutamate or glutamine do not act as substrates or inhibitors. It is less specific for tyrosine; five out of six tyrosine dipeptides were inhibitors and competed with tyrosine.

Since microtubules may form and disappear rapidly while the total cell content of tubulin appears to be constant, it seems unlikely that transcriptional and translational controls could be sufficient to regulate microtubule assembly. Reversible posttranslational modifications are therefore intriguing candidates to modulate the assembly (or function) of microtubules, either by a direct effect on the competence of tubulin to polymerize, or by influencing the partition of tubulin among different cell compartments (Raybin and Flavin, 1976).

An interesting modification has been revealed by work in Caputto's laboratory which showed that brain extracts could incorporate tyrosine into a protein with many similarities to tubulin (Barra et al., 1974), in the absence of tRNA or other components required for protein synthesis. The reaction was reversible in extracts, and the tyrosine appeared to be introduced through a C-terminal peptide bond.

We have previously reported (Raybin and Flavin, 1975, 1976) that the tyrosine is indeed fixed in the α chain of tubulin (tubulin is composed of two very similar chains and α chain is the one migrating more slowly in most sodium dodecyl sulfate–polyacrylamide gel electrophoresis systems). Tubulin was easily separated from a tyrosylating enzyme, called tubulin–tyrosine ligase pending more insight into the nature of the reaction. Tubulin purified by three cycles of in vitro assembly could accept a maximum of 0.25 mol of tyrosine per mol of α chain and could be used as a substrate to assay and purify the enzyme. The 6S tubulin dimer was a substrate, and axonemal doublet microtubules appeared not to be. Tyrosylation did not affect the partition or tubulin between dimers and 36S rings, nor block incorporation of tubulin into microtubules in vitro; it was unclear whether tubulin could assemble without C-terminal tyrosine.

We report here a partial purification of tubulin–tyrosine ligase, and some results concerning the structure of the enzyme, its specificity for substrates and inhibitors, and the reversibility of the reaction which it catalyzes.

Experimental Section

Methods

Analytical Procedures. Tubulin–tyrosine ligase activity was assayed at 37 °C in a final volume of 100 μL in stoppered 6 × 50 mm tubes. Reaction mixtures contained 0.001 to 0.01 unit of ligase and 200 μg of tubulin in 25 mM Tris-HCl[1] (pH 7.2), 30 mM KCl, 12.5 mM $MgCl_2$, 2.5 mM ATP, and 0.1 mM [U-^{14}C]-L-tyrosine (50 mCi/mmol). For optimal activity with purified enzyme, it was necessary to also include 1 mM β-mercaptoethanol and this was done routinely in later experiments. Colchicine, 0.1 mM, was often included but did not affect the results. Reactions were stopped after 20 min by transferring 75 μL of reaction mixture to a Whatman 3MM paper disc which was dropped into a beaker of cold 10% trichloroacetic acid and washed to remove free tyrosine as described by Mans and Novelli (1961). Dried discs were counted in 12 mL of 0.4% (w/v) 2,5-diphenyloxazole in toluene in a Beckman LS 250 counter. A unit of activity is defined as the amount fixing 1 nmol of tyrosine into protein in 1 min, and specific activity is expressed as nmol min^{-1} (mg of protein)$^{-1}$. We have previously reported (Raybin and Flavin, 1975) that tyrosine fixation is proportional to the amount of enzyme and reaction time, and that the ligase is saturated with tubulin at 2 mg/mL. To determine the free tyrosine present in samples of purified tyrosylated [^{14}C]tubulin, or liberated therefrom by carboxypeptidase, total tyrosine was also determined by counting aliquots on unwashed paper discs, or directly in Hydromix (10 mL + 1 mL of water). Free tyrosine was calculated from the difference between total and fixed.

The reverse reaction, tyrosine release from tyrosylated tubulin, was measured under similar conditions (Rodriguez et al., 1973), except that ATP was replaced by 2 mM ADP + 50 mM potassium phosphate, and tubulin and tyrosine were replaced by purified tyrosylated [^{14}C]tubulin (Raybin and Flavin, 1975). Crude extracts were filtered through a column of Sephadex G-50 prior to assay, to remove any free amino acid, and thus ensure that a net detyrosylation was being

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate.

measured and not an exchange of unlabeled for labeled tyrosine. Reactions were stopped by adding cold 10% trichloroacetic acid; the mixtures were placed briefly in a 100 °C bath to flocculate protein, and then cooled and centrifuged 20 min at 35 000*g*. Aliquots of the supernatants, containing free tyrosine, were counted in Hydromix. The amount of free tyrosine could also be determined by filtering out the precipitated protein on a Millipore filter.

Protein was determined by the procedure of Lowry et al. (1951), standardized with bovine serum albumin.

Preparation of Tubulin. Tubulin was prepared by three cycles of assembly by a modification of the method of Shelanski et al. (1973) in which glycerol was either omitted entirely, or present only during the first assembly. Bovine brains, from Frederick County Products, were brought to the laboratory on ice within 1 h of sacrifice. After removing meninges and superficial blood vessels, the cerebral cortex was minced, rinsed with cold deionized water, and suspended in cold homogenization buffer (100 mM Na^+Mes, pH 6.6, 0.5 mM $MgSO_4$, 1 mM each GTP and EGTA): 1.5 mL per g of tissue, or 1.0 mL if glycerol was to be used later. The suspension was homogenized in batches in the cold in a 1-quart Waring blender for 45 s at full speed. The homogenate was centrifuged for 30 min at 27 000*g* (Sorvall GSA rotor) and the supernatant again centrifuged for 1 h at 95 000*g* (Beckman 35 rotor), both at 4 °C. If glycerol was to be added, the final supernatant was mixed with an equal volume of homogenization buffer which was 8 M in glycerol; otherwise solid GTP was added to make the solution 1 mM. The solution was warmed to 37 °C for 20 min to allow microtubule assembly, then centrifuged for 2 h at 25–30 °C at 45 000*g* in a Beckman 21 rotor, or for 1 h at 79 000*g* in Beckman 30 rotors. The microtubule pellets were suspended in cold reassembly buffer (100 mM Na^+Mes, pH 6.6, 0.1 mM $MgSO_4$, 1 mM GTP) with the aid of a type A Dounce homogenizer, kept on ice for 30 min, and centrifuged for 1 h at 4 °C at 113 000*g* (in a Beckman 60Ti rotor). GTP was again added to the supernatant to give a final concentration of 1 mM, and the supernatant was kept at 37 °C for 20 min for a second cycle of microtubule assembly. Microtubules were sedimented at 25–30 °C for 25 min at 79 000*g*. The tubulin was then taken through a similar third cycle of depolymerization and polymerization. The final pellets were dissolved in a minimal volume of cold reassembly buffer and stored at −80 °C at a protein concentration of about 35 mg/mL. Sodium dodecyl sulfate gel electrophoresis showed that the preparation consisted of about 85% tubulin, 12% two high-molecular-weight peptides, and traces of many smaller peptides (Murphy and Borisy, 1975). Since omission of glycerol occasionally gave a poor yield in the second cycle, most of our experiments were done with tubulin prepared with glycerol. One to two hundred milligrams was obtained from 500 g of brain.

Molecular Weight Determination. A 1.5 × 19 cm column of Sephadex G-150 was equilibrated with 25 mM K^+Mes, pH 6.6, 100 mM KCl, and 1 mM each EDTA and mercaptoethanol, under a pressure of 7 cm of water. The column was calibrated with catalase, 250 000; yeast alcohol dehydrogenase, 150 000 (Klotz and Darnell, 1969); pig heart malic dehydrogenase, 70 000 (Thorne and Kaplan, 1963); ovalbumin, 45 000 (Tanford, 1961); and bovine hemoglobin $\alpha\beta$ dimer, 32 000 daltons (Andrews, 1962). The activity assays described in the Worthington Biochemical Corp. Enzyme Manual (1972 edition) were used to determine catalase, alcohol dehydrogenase, and malic dehydrogenase. Hemoglobin and ovalbumin were measured by absorbance at 430 and 280 nm, respectively. In each experiment 0.5 mL was applied to the column and 1-mL fractions were collected. When samples of ligase were analyzed, hemoglobin was always included as an internal standard.

Analytical Polyacrylamide Gel Electrophoresis. The sodium dodecyl sulfate electrophoresis system of Luduena and Woodward (1973, and personal communication), which gives a wide separation of the α and β chains of tubulin, was used for protein samples which had been reduced with dithiothreitol in urea, alkylated with iodoacetic acid, and then heated for 2 min at 100 °C after dialysis against 1% sodium dodecyl sulfate in 10 mM Tris-HCl (pH 8.5). For small samples of protein, it was convenient to use the pH 8.7 sodium dodecyl sulfate–urea system of Eipper (1974), which gives a modest separation of the unmodified α and β chains. The protein was denatured by heating for 3 min at 100 °C in 1% sodium dodecyl sulfate, 1% mercaptoethanol, and 10% glycerol at pH 6.8 (Laemmli, 1970). Analyses were done in 10-cm long cylindrical gels in a Buchler 3-1750 Polyanalyst apparatus, or in ten wells, set in a slab 1.5-mm thick and 8-cm long, in a Bio-Rad Model 220 apparatus. In both cases the electrophoresis compartment was thermostated at 25 °C. Current was applied at 2.5 mA per tube or 35 mA per slab. Gels were stained with Coomassie brilliant blue and destained according to Fairbanks et al., (1971), using 0.05% dye in the first step, 0.0025% in the second, and omitting the third.

Materials

Human apolipoprotein glutamine I was a gift of Dr. Bryan Brewer. Yeast alcohol dehydrogenase, pig heart malic dehydrogenase, catalase, and ovalbumin were from Worthington. DEAE-cellulose, DE-32, was precycled and equilibrated according to the supplier's directions (Whatman). Sephadex G-150 and G-50 were from Pharmacia; agarose (Bio-Gel A-15m) and Coomassie brilliant blue were from Bio-Rad. Avidin, grade VI micrococcal nuclease, type III horse heart cytochrome *c*, octopamine, GTP, cGMP, and cAMP were from Sigma. Amersham/Searle was the source of L-[U-^{14}C]tyrosine and [*side chain*-2-^{14}C]tyramine. Colchicine and L- and D-tyrosine were from Calbiochem; ATP (ultrapure) and bovine hemoglobin from Schwarz/Mann; L-tyrosyl-L-tyrosine, α-L-glutamyl-L-tyrosine, and L-tyrosyl-L-glutamic acid were from Miles Laboratories; L-tyrosine amide, L-alanyl-L-tyrosine, L-tyrosyl-L-alanine, and L-tyrosylglycine were from Vega-Fox biochemicals; tyramine hydrochloride was from Eastman; Hydromix was from Yorktown research.

Results

Purification of Tubulin–Tyrosine Ligase. Tubulin purified by three cycles of assembly was used as assay substrate at saturating levels (2 mg/mL) since we had found it to be free of ligase. Fixation of [^{14}C]tyrosine was proportional to reaction time and enzyme concentration (Raybin and Flavin, 1975) and proved suitable for monitoring purification of the enzyme. All steps were performed at +2 °C.

Four bovine brains (1280 g) were cleaned of meninges, coarsely minced, washed with deionized water, and suspended in an equal volume (v/w) of 25 mM K^+Mes, 1 mM each EDTA and mercaptoethanol, pH 6.6. The supension was homogenized in a Waring blender for 45 s at top speed. The homogenate was centrifuged 30 min at 27 000*g* and the supernatant again centrifuged for 1 h at 95 000*g*. This extract was stable at −80 °C for at least 6 months.

To 840 mL of extract, ammonium sulfate (196 g) was added with stirring. After 20 min the suspension was centrifuged for

TABLE I: Purification of Tubulin Tyrosine Ligase.

Step	Volume (mL)	Total Protein (mg)	Spec Act. (nmol min^{-1} mg^{-1})	Total Units	Yield (%)
Extract[a]	840	13 400	0.027	360	(100)
Ammonium sulfate	100	2 200	0.043	93	25
Sephadex G-50	270	1 300	0.044	59	16
DEAE-cellulose	120	106	0.43	45	12
Concentrated	12	106	0.39	36	10

[a] From 1280 g of brain.

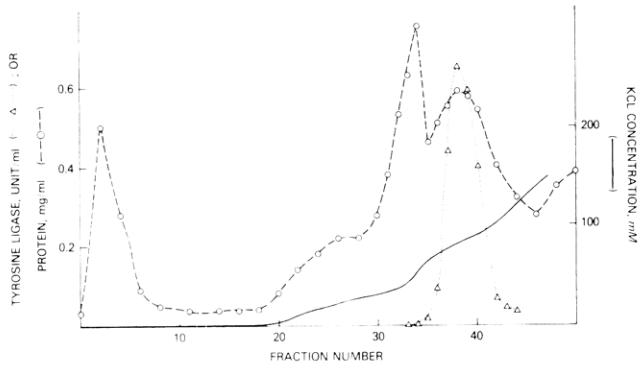

FIGURE 1: Elution of tubulin–tyrosine ligase from DEAE-cellulose.

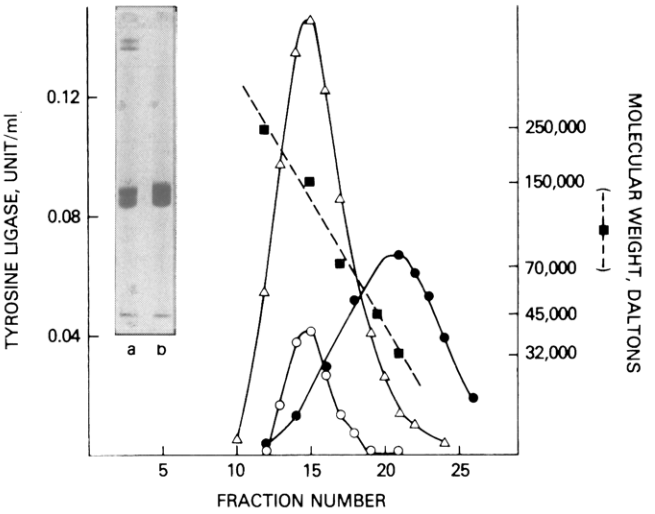

FIGURE 2: Molecular weight of tubulin–tyrosine ligase in extracts, after elution from DEAE-cellulose, and after the latter fraction was mixed with purified 6S tubulin. In separate experiments 0.27 unit of crude brain extract ligase (—○—), 0.75 unit of DEAE-cellulose fraction (—●—), or 0.75 unit of the latter mixed with 50 μg of 6S tubulin (—△—) was applied to the Sephadex G-150 column described in the Methods section. Tubulin was added, in the last case, at 0 °C just before application to the column. Bovine hemoglobin was included as an internal standard in each experiment. The inset is a photograph of polyacrylamide gels (origin at top) showing the composition of the tubulin preparation in the sodium dodecyl sulfate–urea system of Eipper (1974): (b) 56 μg of 6S tubulin from an agarose A-15m column (Raybin and Flavin, 1975); (a) 51 μg of the tubulin, purified by three cycles of assembly, from which b was derived. The proteins used for molecular weight calibration (- -■- -) are described in the Methods section.

20 min at 20 000*g*. Ammonium sulfate, 71 g, was added to the supernatant, and the precipitate (collected by centrifugation) was dissolved in 100 mL of the above buffer and desalted on a 5 × 60 cm column of Sephadex G-50 (medium) equilibrated with the same buffer; at this stage ionic strength did not affect recovery from Sephadex. This fraction was unstable ($t_{1/2}$ a few days at +4 or −80 °C) both before and after desalting, and the yield was invariably low (Table I). The low yield did not appear to be due to resolution of the enzyme activity into more than one component since recombination of low and high salt fractions at the periphery of the activity peak yielded only additive amounts of activity. Activity was not enhanced by adding boiled extract.

The desalted fraction was immediately applied to a 2.5 × 37 cm DEAE-cellulose column, preequilibrated with the above buffer, and eluted with 450 mL of buffer, then with a KCl gradient (500 mL each, of 0 and 250 mM, in buffer); 20-mL fractions were collected. The active fractions (Figure 1) were concentrated by ultrafiltration through an Amicon XM-50 or PM-30 membrane. This DEAE fraction was slightly less stable than the extract when stored at −80 °C. Optimal activity required addition of 0.5 mM mercaptoethanol to the reaction mixture. No activity could be recovered from agarose or Sephadex columns (see below) unless the buffer contained 100 mM KCl. The specific activity of the extract was often 0.05; then that of the final fraction was 0.9. Extract activity was often low if EDTA was omitted. The activity was very sensitive to heat (Raybin and Flavin, 1975) and was variably lost on dialysis (60% in 4 h) at all stages.

Because of the poor yield in the precipitation step, further purification was not attempted, but alternative first steps were tried. If the viscous extract was applied directly to DEAE-cellulose, the result was unsatisfactory. Ammonium sulfate fractionation of liver extract gave only slightly better yields. Acetone fractionation of brain extract gave a 30% yield spread over many fractions. Ligase precipitated from brain extract at pH 4.8 with a threefold purification but would then no longer redissolve at neutral pH.

Tubulin Alters the Apparent Size of the Ligase. A possible clue to the difficulty in purifying the enzyme was suggested by studying its molecular size. Gel filtration showed an apparent molecular weight for the ligase of 150 000 in crude extracts (Raybin and Flavin, 1975) and the ammonium sulfate fraction,[2] but only 35 000 in the DEAE fraction (three different ones were tested). When DEAE and extract fractions were mixed before applying them to a Sephadex G-150 column, we found unexpectedly that all the activity eluted in the 150 000-dalton peak. An Amicon PM-30 ultrafiltrate of extract did not alter the size of the DEAE ligase. However, when DEAE ligase was mixed, just before application to the column, with its tubulin substrate (purified by three cycles of assembly), all the activity was in the larger form of the enzyme. Agarose 15m columns separate tubulin into two fractions, a "36S" fraction of oligomeric rings which contains all of the two high-molecular-weight peptides (inset, Figure 2) and a "6S"

[2] T. Kobayashi (unpublished results) finds that simply keeping ligase at pH 7 for 30 min at 0 °C in 50% saturated ammonium sulfate inactivates extract 70%, but DEAE fraction 95%. Ammonium sulfate probably dissociates ligase to the labile 35 000-dalton species, which reassociates with tubulin on dilution. DEAE-cellulose chromatography yields the 35 000-dalton species only when the ligase fraction applied is devoid of free native tubulin, as judged by colchicine binding. If the ligase fraction has been kept in a buffer which stabilizes tubulin, the eluted activity has a molecular size of 150 000, and it elutes only with high salt, in the same fractions containing free tubulin. This confirms that the larger species is a complex with tubulin, and not an oligomer of the 35 000-dalton enzyme.

TABLE II: Kinetic Constants for Tyrosine and Related Compounds in the Tubulin–Tyrosine Ligase Reaction.

Substrate or Inhibitor	K_m (mM)	K_i (mM)
L-Tyr	0.043	
L-Phe[a]	2.9	3.3
L-Phe((OH)$_2$)[a]	0.16	0.20
Tyramine	8.0	2.1
L-Tyr-L-Tyr		0.023
α-L-Glu-L-Tyr		0.16
L-Ala-L-Tyr		0.10
L-Tyr-L-Ala		0.19
L-Tyr-L-Gly		0.23
L-Tyr-L-Glu	(nonlinear plot)	

[a] Data from Rodriguez et al. (1975).

fraction of nearly pure tubulin dimers (Weingarten et al., 1974). Figure 2 shows that addition of a small amount of this 6S fraction also converted the DEAE ligase completely to the larger form. In another experiment we purified dimeric tubulin by centrifuging a three-cycle preparation at 150 000*g* for 140 min at 2 °C and passing the supernatant through a phosphocellulose column (Kirschner et al., 1974). When 20 μg was mixed with 1.2 units of DEAE ligase, about half the activity was converted to the higher molecular weight form. We calculate that, if all the tubulin were bound to half of the ligase in 1:1 stoichiometry, the ligase would comprise $\frac{1}{250}$ of the protein in the DEAE fraction, or $\frac{1}{5000}$ of the protein in a crude extract.

Ligase and free tubulin are widely separated by DEAE chromatography which might thus be expected to dissociate a complex of the two. Recovery of activity added to the G-150 column (Figure 2) was 96% for extract, 72% for DEAE fraction, and 108% for the latter when mixed with tubulin. Thus tubulin stabilized the ligase during filtration. We do not know whether the 35 000-dalton protein has any activity itself (see Discussion). The sum of the molecular weights of tubulin (110 000) and the DEAE ligase is 145 000, compared with 150 000 for the ligase mixed with tubulin. It is most likely that ligase binds tubulin in 1:1 stoichiometry, though we cannot exclude the possibility that tubulin induces the formation of ligase oligomers.

Probably unrelated to the above, we found that the ligase activity of either extract or DEAE fraction was enhanced about threefold by adding to it the macromolecular fraction obtained by Sephadex filtration of an extract of *Tetrahymena* (ATCC 10542). Enhancement was proportional to the amount of *Tetrahymena* extract, which itself had no ligase activity. Equivalent amounts of bovine serum albumin did not affect ligase activity. The molecular weight of the DEAE ligase remained 35 000 after mixing with *Tetrahymena* extract.

Reversal of the Tubulin–Tyrosine Ligase Reaction. Rodriguez et al. (1973) showed that brain extract would liberate free [^{14}C]tyrosine from protein, when ATP was replaced by ADP + P_i. We measured the rate of detyrosylation (Methods section) using tubulin purified free of ligase by three cycles of assembly, after 7% of the α chains had been tyrosylated. The substrate concentration used was 1.2×10^{-6} M (10^{-7} M fixed [^{14}C]tyrosine); there was insufficient substrate to test whether this was saturating. With both crude extract and DEAE fraction of ligase, the rate was proportional to the amount of enzyme fraction added, and to reaction time, until slightly more than half the [^{14}C]tyrosine had been released, after which the rate declined sharply. Using the proportional range to assay the detyrosylating activity, we confirmed the requirements for ADP and P_i; 50 mM potassium phosphate gave maximal rates, and arsenate was half as effective. Comparison of the rates of detyrosylation by extract and a 17-fold purified DEAE fraction showed the respective rates to be 0.078 and 0.087 nmol min^{-1} unit^{-1} of ligase. The nearly constant ratio of activities suggests tyrosine removal and addition are catalyzed by the same enzyme. The detyrosylation rate appeared to be $\frac{1}{12}$ of the tyrosylating rate. This value is not meaningful; for example, the rates would be equal if 93% of the substrate α chains had C-terminal [^{12}C]tyrosine. Because of uncertainty about the extent to which tubulin as isolated is already tyrosylated, and about the fraction of it which is actually available as a substrate for the ligase, we could not determine a K_m for tubulin. The available evidence suggests the K_m may be <2 μM.

Substrates and Inhibitors. The 6S tubulin dimer is unequivocally a substrate; the 36S ring fraction was also tyrosylated, but is in equilibrium with dimers (Raybin and Flavin, 1975). The rate and extent of tyrosylation are unaffected by 0.1 mM colchicine or 2 mM Ca^{2+}. We have been unable to test whether microtubules assembled in vitro are a substrate because the high Mg^{2+} concentration required by the ligase is unfavorable for assembly. Tyrosine was not fixed into axonemal outer doublet microtubules, but the latter also inhibited fixation into soluble tubulin (Raybin and Flavin, 1975).

In brain extracts the only peptide which is tyrosylated is the α chain of tubulin. The C-terminal residue to which tyrosine is linked is either Glu or Gln (Arce et al., 1975). Since tubulin is present in higher concentration than other proteins in these extracts, we have begun to test whether, at high concentrations, other proteins with either of these C-terminal residues can serve as substrates or inhibitors. Horse heart cytochrome *c* and avidin, with C-terminal Glu (Margoliash and Smith, 1961; DeLange and Huang, 1971), and micrococcal nuclease and apolipoprotein-glutamine I, C-terminal Gln (Cusumano et al., 1968; Baker et al., 1975), were added to assay mixtures containing ligase ± brain tubulin. The amounts added were in 33-, 10-, 6-, and 8-fold molar excess, respectively, over the saturating tubulin concentration. None appeared to be inhibitors or substrates.

Phenylalanine and dihydroxyphenylalanine [Phe((OH)$_2$)] have been shown to be substrates for the ligase, and also to competitively inhibit tyrosine fixation (Rodriguez et al., 1975); neuroactive amines related to tyrosine did not inhibit, and we found the same to be true for DL-octopamine (25 mM) and serotonin (12 mM). L-Tyrosineamide and D-tyrosine also did not inhibit. Tyramine inhibited, and [^{14}C]tyramine was fixed into protein (Table II) by DEAE ligase fraction; however, K_i did not equal K_m and we have not yet shown that it was fixed into tubulin. Inhibition by tyrosine dipeptides was studied by assaying tyrosine fixation at five different tyrosine concentrations (confirmed by absorbance measurement), at each of three dipeptide concentrations. Illustrative data are shown in Figure 3. Five out of six dipeptides were competitive inhibitors (Table II).

Addition of 10^{-5} M cAMP or cGMP, in addition to ATP, to ligase reaction mixtures, did not affect the rate or extent of tyrosine fixation.

Discussion

Tubulin–tyrosine ligase has an apparent molecular weight of 150 000 as it is extracted from brain and after ammonium sulfate fractionation, but 35 000 after ion-exchange chromatography which separates it from free tubulin. The molecular

weight is restored to 150 000 by adding small amounts of 6S dimeric tubulin (mol wt 110 000). The large form could be a tubulin induced tetramer of the small form, but is much more likely to be a stoichiometric complex of one tubulin substrate and one 35 000-dalton "enzyme". Is the 150 000-dalton form the actual enzyme–substrate complex, ES, or does ES involve a second tubulin molecule? The catalytic K_m for tubulin has not been adequately determined, but is probably <2 mM, and might be low enough to account for the stability of the 150 000-dalton enzyme. The question may be answered when the dissociation constant of the latter can be compared with the K_m. If tyrosylation modulates tubulin assembly or function, one might expect the tyrosylating enzyme in turn to be controlled in some way. Thus it would be interesting if the 150 000-dalton enzyme were not an ES complex, as this might suggest activation by association with tubulin or a specific subspecies of tubulin. The 35 000-dalton enzyme is converted to the larger form as soon as it is exposed to tubulin, even at 0 °C; to determine whether it has catalytic activity, it would be necessary to find a substrate other than, or a partial reaction not involving, tubulin. The easily reversible interconversion of the two enzyme species should now facilitate purification.

The enzymes catalyzing addition and removal of tyrosine fractionated together during a 20-fold purification and are probably the same protein. Nothing is known about the stoichiometry or mechanism of either reaction. Chromatographic identification of radioactive α-glutamyltyrosine in partial acid hydrolysates (Arce et al., 1975) constitutes the principal evidence that the tyrosine is introduced through a C-terminal peptide bond. Our results indicate that tubulin–tyrosine ligase catalyzes both addition and removal of tyrosine, but do not show that it is only enzyme that can remove tyrosine, and there may be carboxypeptidases in brain extracts also capable of doing so.

The ligase appears very specific for tubulin. Tyrosine was not fixed into any other protein in crude brain extracts, or when tubulin was replaced by large amounts of other purified proteins having C-terminal glutamate or glutamine. The inability of the latter proteins to serve as substrates was only determined with the 35 000-dalton enzyme species; since we do not know whether this is an active form of the enzyme, the experiment should be repeated with the 150 000-dalton species. The ligase was less specific for tyrosine, as five out of six tyrosine dipeptides were effective inhibitors and appeared to compete with tyrosine, the exception being tyrosylglutamate with the sequence inverted from that in tyrosylated tubulin (Table II). Dipeptides with C-terminal tyrosine (analogues of tyrosylated tubulin) were slightly more effective than those with N-terminal tyrosine. We have not tested whether the peptides compete with tubulin or tyrosylated tubulin.[3] These results suggest that affinity chromatography may be useful in purifying the enzyme, and that the natural substrate need not necessarily be tyrosine itself.

A preliminary titration of the amount of tubulin needed to convert the 35 000- to the 150 000-dalton form of the ligase suggested that the ligase constitutes somewhere about $1/5000$ of the protein in the high-speed supernatant of crude brain extract. If this figure is correct, the pure 35 000-dalton enzyme would have a specific activity of 250 nmol min^{-1} mg^{-1} and a turnover number of 10, and the molar ratio of enzyme to substrate in extracts would be 1:150. This proportion of enzyme is quite high, and in fact when fresh rat brain extract was incubated with [^{14}C]tyrosine the endogenous tubulin was rapidly tyrosylated and the reaction came to a stop in 10 min (Raybin and Flavin, 1975). Thus enzyme is in excess over tubulin in extracts, and since the concentration of free tyrosine in brain exceeds the K_m concentration for the enzyme (Rodriguez et al., 1975), it appears that brain tubulin *could* be mostly tyrosylated in vivo. We will report elsewhere (Raybin and Flavin, *J. Cell Biol.*, in press), on the extent to which cytoplasmic brain tubulin is tyrosylated in vivo, and on the effect of tyrosylation on microtubule assembly in vitro.

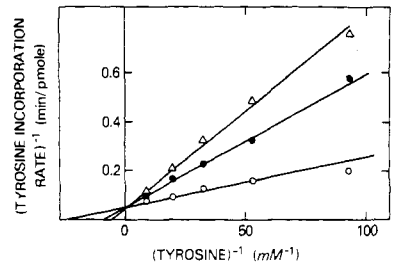

FIGURE 3: Competitive inhibition of tyrosine fixation by α-L-glutamyl-L-tyrosine. DEAE ligase fraction was incubated under the standard assay conditions (except for the tyrosine concentration) with (○) no inhibitor, (●) 0.2 mM, and (△) 0.5 mM inhibitor.

Acknowledgments

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[3] Recently we have obtained a synthetic octapeptide, Gly-Glu-Glu-Glu-Gly-Glu-Glu-Glu, corresponding to the C-terminal sequence of the untyrosylated α chain (Lu and Elzinga, 1976, and personal communication). The DEAE fraction of ligase does not tyrosylate this peptide, or catalyze an exchange of tyrosine into the corresponding nonapeptide with C-terminal tyrosine. The peptides were much less effective in inhibiting the tyrosylation of tubulin (T. Martensen and T. Kobayashi, unpublished results) than the tyrosine dipeptides listed in Table II.

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Thermodynamics of Binding to Native α-Chymotrypsin and to Forms of α-Chymotrypsin in Which Catalytically Essential Residues Are Modified; a Study of "Productive" and "Nonproductive" Associations†

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ABSTRACT: The standard free energy ($\Delta G°$), enthalpy ($\Delta H°$), and entropy ($\Delta S°$) of association for proflavin and D- and L-*N*-AcTrp have been obtained at pH 7.8 for native α-chymotrypsin (Cht) and for forms of Cht in which essential catalytic residues of the active site are modified. The modified Cht forms studied are dehydroalaninyl-195-α-Cht and *N*-methylhistidinyl-57-α-Cht. Associations to native Cht (pH 7.8) are characterized by negative $\Delta H°$ and $\Delta S°$ values (i.e., for L-AcTrp $\Delta H° = -9.1$ kcal/mol and $\Delta S° = -21$ eu at $T = 25$ °C). In contrast, we found associations to modified Chts to be characterized by an enthalpy near zero and a positive entropy of association, the values of the $\Delta H°$ and $\Delta S°$ for association to the modified Cht forms being similar to those expected for transfer of small aromatic molecules from water to a nonpolar solvent phase. Differences in $\Delta H°$ and $\Delta S°$ observed for binding of substrate analogues and inhibitors to modified and native Cht (pH 7.8) are approximately +10 kcal/mol and +30 eu, respectively. Data from D. D. F. Shiao ((1970), *Biochemistry 9,* 1083) similarly show differences of comparable magnitude between binding of substrate analogues to active α-Cht (pH 7.8) and the His-57 protonated form of α-Cht (pH 5.6). The negative $\Delta H°$ and $\Delta S°$ values of associations for binding to active α-Cht indicate that a substrate-induced conformational change occurs on substrate association with the primary binding site (S_1), which does not occur in Ser-195 and His-57 modified Cht. From these differences we infer a linkage between binding of substrate into S_1 and the catalytic residues in the nucleophilic subsite (S_1–S_1'). Our data also show that associations of substrate analogues into potentially productive Michaelis complexes S_1 cannot be easily differentiated from associations that are nonproductive (i.e., nonactivated) from their $\Delta G°_{obsd}$, but may be differentiated by their respective $\Delta H°_{obsd}$ and $\Delta S°_{obsd}$ for association. Accordingly, it is indicated that the probable substrate *association–activation* process, characterized thermodynamically in this work, occurs in the substrate binding step and leads to lowered free energies of activation in catalytic steps succeeding binding; however, the process does not influence the observed strength of substrate binding.

The concepts of induced fit, substrate distortion, and productive substrate orientation have received wide support as mechanisms that may make a significant contribution toward lowering reaction activation energies in enzyme-catalyzed reactions (Jencks, 1975). In most of these hypotheses, it is argued that a favorable free energy of productive substrate binding into the noncovalent Michaelis complex is partially utilized to lower the activation energy of succeeding covalent bond making or breaking steps in the enzymic mechanism. This is accomplished by either (i) a conformational change in the enzyme active site induced by substrate binding leading to a precise orientation of catalytic residues of the enzyme and the reactive bond in the substrate (Koshland and Neet, 1968), (ii) distortion of the reactive bonds of the substrate on binding toward the transition state for the enzyme-catalyzed reaction (Eyring et al., 1954; Jencks, 1969a), or (iii) simply by the restriction of translational and rotational degrees of freedom in the substrate on binding with respect to the catalytic residues of the enzyme active site (Storm and Koshland, 1970; Bruice, 1970; Page and Jencks, 1971). These mechanisms of substrate and/or enzyme activation toward catalysis occurring in the noncovalent Michaelis complex between enzyme and substrate will be referred to in the following discussion as association–activation mechanisms. More precisely, association–activation describes the activation of substrate and/or enzyme toward catalysis on association with an enzyme active site prior to any covalent bond making or breaking step in the enzymic mechanism. The term encompasses both orientation and substrate distortion hypotheses.

In the case of chymotrypsin there is evidence in support of all three types of association–activation mechanisms (types

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