

Purification of Brain Tubulin-Tyrosine Ligase by Biochemical and Immunological Methods

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ABSTRACT Tubulin-tyrosine ligase (TTL), the enzyme responsible for the reversible addition of a tyrosine residue at the carboxyl end of α -tubulin, has been purified from porcine brain using a purification scheme based on standard biochemical procedures. The enzyme preparation was nearly homogeneous (purity >95%), was free of tubulin, and could be stored in the presence of glycerol for several months without loss in activity. To develop a more convenient purification of TTL, we have isolated mouse hybridoma cells secreting antibodies to TTL. These monoclonal antibodies recognize TTL not only in brain tissue but also in the liver of various mammals. Monoclonal antibodies isolated from ascites fluid allowed a rapid purification of TTL from a crude brain extract. TTL stayed bound to the immunoaffinity column in 1.5 M NaCl and was eluted with 3 M MgCl₂. Highly active TTL was recovered nearly quantitatively at >95% purity and could be stabilized in the presence of glycerol. Glycerol gradient centrifugation, SDS gel electrophoresis and immunoblots identified TTL as a monomeric protein with an apparent polypeptide molecular weight of about 40,000. A one to one complex of TTL with $\alpha\beta$ -tubulin was observed by gradient centrifugation.

Due to its turnover in vivo and in vitro the carboxyterminal tyrosine of α -tubulin seems to play a special role in the tubulin $\alpha\beta$ -heterodimer (for a review, see reference 28). Although encoded by the messenger RNA (30), the tyrosine residue can be removed by a presumptive tubulin-tyrosine carboxypeptidase (1, 9, 15), which has so far not been characterized in detail. Tyrosination is again achieved by the enzymatic addition of tyrosine to the carboxyterminal glutamate of detyrosinated α -tubulin (2, 22). The enzyme responsible for this process, termed tubulin-tyrosine ligase (TTL),¹ is highly specific for tubulin (23) and is found in various vertebrate (21, 22) and invertebrate tissues (8). It requires Mg²⁺ and K⁺ with tyrosine, and ATP and detyrosinated tubulin acting as substrates (4, 23). Partial purification of TTL from bovine brain requires several steps (23), while a nearly homogeneous preparation can be obtained by phosphocellulose (PC) chromatography and two successive steps using tubulin and ATP affinity chromatographical procedures (18). The resulting preparation is unfortunately rather unstable (4), thus limiting its use in

biochemical studies of the tubulin tyrosination-detyrosination cycle, the physiological function of which is still unknown.

Here we describe a biochemical purification procedure that yields an essentially homogeneous TTL preparation. If the enzyme is stabilized by the addition of glycerol, it can be stored for several months without loss in activity. A more convenient and very rapid purification procedure based on immunoaffinity chromatography is also described. This is based on mouse monoclonal antibodies that we have raised against TTL from porcine brain.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: L-[3,5-³H]tyrosine (specific activity, 51 Ci/mmol) and L-3,4-dihydroxy[ring-2,5,6-³H]phenylalanine (22 Ci/mmol) from The Radiochemical Centre, Amersham, England; L-[alanine-2,3-³H]phenylalanine (21 Ci/mmol) and [³H(G)]tyramine (*p*-hydroxyphenylethylamine) (35.7 Ci/mmol) from New England Nuclear, Boston, MA; L-tyrosine from E. Merck, Darmstadt, Federal Republic of Germany (FRG); PIPES from Calbiochem, Giessen, FRG; EGTA, L-phenylalanine, L-3,4-dihydroxyphenylalanine (L-DOPA), *p*-hydroxyphenylethylamine (tyramine), phenylmethylsulfonyl fluoride, and carboxypeptidase A (bovine pancreas; type II-PMSF; 48 U/mg) from Sigma, St. Louis, MO; ATP and GTP from Waldhof, Mannheim, FRG; DEAE-cellulose (pre-swollen DE52), phosphocellulose (P11), and Whatman 3MM

¹ *Abbreviations used in this paper:* DTT, dithiothreitol; PC, phosphocellulose; PMSF, phenylmethylsulfonyl fluoride; TTL, tubulin-tyrosine ligase.

filter paper from Whatman, Maidstone, England; CNBr-activated Sepharose 4B, DEAE-Sepharcel, and AH-Sepharose 4B from Deutsche Pharmacia, Freiburg, FRG; polyethyleneglycol, type 20,000, from Serva, Heidelberg, FRG; protein standards from Boehringer, Mannheim, FRG.

Methods

Pig brain microtubule protein was isolated by three cycles of temperature-dependent assembly/disassembly according to Shelanski et al. (25) in 100 mM PIPES (pH 6.5), 1 mM MgSO₄, 2 mM EGTA, 1 mM GTP and 1 mM 2-mercaptoethanol. In the first cycle of polymerization, glycerol and phenylmethylsulfonyl fluoride (PMSF) were added to 4 M and 0.2 mM, respectively. Homogeneous tubulin (PC-tubulin) was prepared from microtubule protein by PC chromatography as described (6). The purified proteins were stored in aliquots at -70°C. Detyrosinated microtubule protein and tubulin were obtained by treatment of microtubule protein with pancreatic carboxypeptidase A as described (15).

The rat monoclonal antibodies to α -tubulin of yeast used in this study, clone YL 1/2, were a kind gift from Dr. J. V. Kilmartin, Cambridge, UK (7). YL 1/2 has been shown to react only with the tyrosinated form of the α -subunit of mammalian brain tubulin (31).

Protein purity was checked by polyacrylamide slab gels in the presence of SDS (SDS-slab gels) using the method of Laemmli (11). Protein transfer from gels to nitrocellulose sheets was done essentially by the method of Towbin et al. (29). After incubation of the nitrocellulose sheets with mouse monoclonal antibody to pig TTL, antibody decoration was visualized by treatment with peroxidase-labeled rabbit anti-mouse antibody (DAKO, Copenhagen, Denmark) followed by incubation with substrate.

Sepharose 4B-sebacic acid hydrazide-ATP (27.3 μ mol ATP/ml gel) was prepared as previously described (12). L-Tyrosine was linked to 6-aminoethyl-Sepharose 4B by carbodiimide coupling using *N*-ethyl-*N'*-(3-dimethylamino-propyl)carbodiimide hydrochloride as described (4). Protein concentrations were determined by the Lowry reagents (13) using bovine serum albumin as standard. The sedimentation coefficient of TTL was determined by glycerol gradient centrifugation.

TTL Assay

Unless stated otherwise, the TTL reactions were carried out in 50- μ l assays containing 25 mM K⁺MES (pH 6.8), 150 mM KCl, 12.5 mM MgCl₂, 2.5 mM ATP, 1 mM dithiothreitol (DTT), 150 μ g microtubule protein, 100 μ M L-[³H]tyrosine (specific activity, 4.5 \times 10⁵ dpm/nmol), and 10 μ l enzyme (maximally 0.01 units). For certain assays carboxypeptidase A-treated microtubule protein was used. After incubation for 0–30 min at 37°C, aliquots of 30 μ l were taken and placed on Whatman 3MM paper disks which were processed as described (14). Dried disks were counted in 10 ml Insta-Fluor (Packard Instrument Co., Inc., Downers Grove, IL). One unit of enzyme is defined as the amount that incorporates 1 nmol of tyrosine into microtubule protein in 1 min under the conditions used for the reaction.

Standard Purification of TTL without Immunoaffinity

The initial steps (I–III, see also Table I) followed essentially the procedure of Murofushi (18). Unless indicated otherwise, all purification steps were done at 4°C. 1 kg pig brain was homogenized in a Waring Blender after addition of 600 ml 10 mM K⁺MES (pH 6.8, containing 50 mM KCl, 0.5 mM MgCl₂, 1 mM EGTA, 1 mM ATP; buffer A). The crude extract obtained after centrifuga-

tion (30,000 g, 1 h) was mixed with an equal volume of buffer A supplemented with 8 M glycerol. After incubation for 40 min at 37°C, microtubules were removed by centrifugation (100,000 g, 40 min, 30°C) and the supernatants were collected (fraction I). 400 ml DEAE-cellulose (DE 52) equilibrated with 25 mM K⁺MES buffer (pH 6.8, 5 mM MgCl₂, 0.5 mM DTT; buffer B) were added to fraction I that had been diluted with half a volume of buffer B. After mixing for 2 h the DEAE-cellulose was collected, resuspended in 400 ml of 25 mM K⁺MES buffer (pH 6.8, 50 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT) and filled into a column (5 \times 30 cm). Proteins were eluted with a KCl gradient from 0.05 to 0.3 M containing 25 mM K⁺MES (pH 6.8), 5 mM MgCl₂, 0.5 mM EDTA, and 0.5 mM DTT (2 liters of total volume). Fractions of 10 ml were collected at \sim 2 ml/min. Ligase eluted at \sim 0.2 M KCl (fraction II). After clarifying by centrifugation fraction II was passed at a rate of 40 ml/h through a Sepharose 4B-sebacic acid hydrazide-ATP column (1.5 \times 6 cm) equilibrated with 25 mM K⁺MES buffer (pH 6.8, 5 mM MgCl₂, 0.5 mM DTT). After washing with the same buffer containing first 500 mM KCl (300 ml) and then 100 mM KCl (50 ml), the enzyme was eluted with 25 mM K⁺MES buffer containing ATP (pH 6.8, 100 mM KCl, 25 mM ATP, 25 mM MgCl₂, 0.5 mM DTT) (fraction III). Fraction III (90 ml) was concentrated in dialysis bags with polyethylene glycol 20,000 to 10.3 ml. For the remaining steps see Results and Table I.

Antibody Production

IMMUNIZATION: Three female BALB/c mice 6–8 wk in age were immunized at intervals of 3 wk with purified ligase (10–20 μ g protein/injection; fraction V of Table I) using Freund's complete adjuvant for the first injection and incomplete adjuvant for the two subsequent injections. Sera were tested in the peroxidase spot test (5) against the original antigen and in the enzyme test described below. All test sera reacted at least at a 1:1,000 dilution in the spot test.

FUSION AND LARGE SCALE PRODUCTION OF ANTIBODIES: Spleen cells from the mouse giving the strongest reaction were fused with cells from the myeloma line PAI (26) using the general procedure described in reference 3. After fusion, cells were aliquoted into thirty 24-well plates using HAT medium. Medium was changed twice a week. Macroscopic colonies were visible by day 10. Positive colonies were cloned twice in soft agar. Ascites fluids were produced in female BALB/c mice. After precipitation with 50% ammonium sulfate, dialysis against 10 mM Na-phosphate, pH 7.4, and loading on to DEAE-Sepharcel, IgGs from ascites fluids were eluted with 40 mM Na-phosphate, pH 7.4.

ENZYME ASSAY FOR SPECIFIC ANTIBODIES: Affinity-purified sheep anti-mouse IgGs were coupled to CNBr-activated Sepharose 4B (\sim 3 mg IgG/ml gel) and stored in PBS containing 1% BSA (wt/vol) and 0.02% NaN₃.

10–20 μ l of sedimented Sepharose was added to 0.3 ml colony supernatant or 0.3 ml diluted serum (1:100 diluted with PBS) in small narrow Eppendorf tubes, which were slowly rotated for 2 h at room temperature. After sedimentation the Sepharose was washed three times with modified ligase buffer (25 mM K⁺MES, pH 6.8, 100 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM ATP, 0.5 mM DTT, 10% glycerol, 1% BSA [wt/vol], 0.02% NaN₃). After sedimentation and aspiration of excess buffer 15 μ l ligase (fraction V) were added to the Sepharose and the tubes were rotated for 1 h in the cold room. After sedimentation of the Sepharose, 10 μ l of the supernatant were tested for the enzyme activity using CPA-treated microtubule protein as substrate. After incubation for 30 min at 37°C, 1 μ l of the ligase reaction mixture was spotted on to nitrocellulose and the peroxidase spot test was performed with the rat monoclonal α -tubulin antibody (clone YL 1/2) specific for tyrosinated α -tubulin (31).

TABLE I
Purification of Tubulin-Tyrosine Ligase From Pig Brain

Fraction	Purification step	Total protein mg	Total enzyme activity		Specific activity U/mg	Yield %	Purification -fold
				U			
I	37°C	9,420		1,040	0.11	100	1
II	DEAE-cellulose	1,472		997	0.68	96	6
III	Sepharose 4B-sebacic acid hydrazide-ATP	21.0		349	16.6	34	150
IV	Glycerol gradient	9.1		380	42	37	380
V	PC	0.14		107	762	10.3	6,930
VI	Tyrosyl-aminoethyl-Sepharose 4B	0.05		16.2	324	1.6	2,950

A preparation procedure, starting from 1,000 g of pig brain, is given. For further details see text.

ENZYME ASSAY WITH IMMOBILIZED LIGASE: Nitrocellulose disks (5 mm in diameter) were incubated with 0.5 ml monoclonal ligase antibodies purified from ascites fluids (see above) or unspecific mouse IgGs (1 mg/ml in PBS) for 30 min at room temperature. The disks were then saturated with BSA-PBS (1% BSA [wt/vol] in PBS), washed with modified ligase buffer (see above) containing 1% BSA and incubated with partially purified ligase (0.5 ml/disk of fraction IV) for 30 min on ice. After extensive washing with modified ligase buffer, the disks were incubated with 50 μ l reaction mixture and incorporation of [3 H]tyrosine was measured as described above.

IMMUNOAFFINITY CHROMATOGRAPHY: Approximately 10 mg of purified ascites IgGs were coupled to 1 g of CNBr-activated Sepharose 4B. For large scale preparations pig brain extracts were prepared essentially as described in 25 mM K⁺MES extraction buffer (pH 6.8, 100 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM ATP, 0.5 mM DTT, 0.2 mM PMSF, 10% glycerol [vol/vol]). After centrifugation for 45 min (20,000 g, 4°C), the supernatants were warmed to 37°C for 40 min and centrifuged for 40 min (100,000 g, 30°C). The supernatants were incubated overnight with the immobilized antibody (1 ml Sepharose gel was used for 50 ml extract) in the cold room and the following steps were done in the cold. The Sepharose was extensively washed with extraction buffer without PMSF, centrifuged two times through 1 M sucrose in extraction buffer to remove aggregates, and then washed with extraction buffer plus 1.5 M NaCl. After repeated washing with extraction buffer alone, the enzyme was eluted with 3 M MgCl₂ in extraction buffer.

RESULTS

Standard Purification of TTL by Biochemical Methods

After elution from the Sepharose-sebacic acid hydrazide-ATP column (fraction III; see Materials and Methods and Table I) ligase-positive fractions were concentrated with polyethylene glycol. Further purification was achieved by glycerol gradient centrifugation, which also stabilizes the enzyme (see recovery in Table I). Samples were run on a linear 10–20% (vol/vol) glycerol gradient in 25 mM K⁺MES buffer (pH 6.8, 20 mM KCl, 1 mM MgCl₂, 1 mM DDT) for 24 h at 4°C (at 27,000 rpm, 131,000 g, in a Beckman SW27 rotor [Beckman Instruments, Palo Alto, CA]). Active fractions were pooled (fraction IV), dialyzed against buffer C (25 mM K⁺MES, pH 6.8, 1 mM DTT, 1 mM EDTA, 0.5 mM ATP, 0.5 mM MgCl₂ and 20% [vol/vol] glycerol) and applied to a PC (P11, Whatman) column (2 \times 6 cm) in the same buffer. After washing first with buffer C (50 ml) followed by 20 ml 25 mM K⁺MES buffer (pH 6.8, 200 mM KCl, 1 mM EDTA, 1 mM DTT, 20% [vol/vol] glycerol) and then by buffer C (20 ml) again, the enzyme was eluted with 25 mM K⁺MES buffer (pH 6.8, 25 mM ATP, 25 mM MgCl₂, 1 mM DTT, 20% [vol/vol] glycerol) (fraction V). Although this preparation had a very high specific activity (760 U/mg; Table I) gel electrophoresis revealed in addition to a major band at 40,000, which accounts for ~80% of the protein, several minor bands of higher

apparent molecular weights (Fig. 1, slot 1). To remove these impurities, we used a further substrate affinity chromatography.

Fraction V was dialyzed against binding buffer (25 mM K⁺MES, pH 6.8, 20 mM KCl, 1 mM MgCl₂, 1 mM DTT, 20% [vol/vol] glycerol) and loaded on to a L-tyrosyl-amino-hexyl-Sepharose 4B column (1.5 \times 10 cm) equilibrated with the same buffer. After washing with binding buffer, the salt concentration was raised stepwise first to 100 and then to 400 mM KCl. During the 400 mM KCl step the glycerol concentration was raised to 40% (vol/vol). Ligase eluted in the last step. Although this pool (fraction VI) had a somewhat lower specific activity (325 U/mg) than fraction V, SDS gel electrophoresis revealed only a single band with a molecular weight of 40,000 (Fig. 1, slot 2).

Ligase purification by the standard procedure is rather cumbersome because of the low level of enzyme present in the starting material and the relative loss of total activity encountered in steps III, V, and particularly VI (Table I). The most active fraction (V) required a 7,000-fold purification and was obtained in only 10% yield in respect to the total activity present in the first warm supernatant. Since subsequent substrate affinity chromatography leads to poor recovery of activity and a more than twofold loss in specific activity (see Table I for fraction VI) most biochemical studies have been concentrated on the slightly less pure fraction V. That the major protein of 40,000 seen in this fraction is indeed TTL is shown by immunological experiments (see below).

Using the purification procedure of Murofushi (18), we did not succeed in obtaining ligase free of two minor contaminating polypeptides migrating at *M_r* 42,000 (possibly actin) and 55,000 (possibly tubulin) in SDS-slab gels. The first contaminant could be removed from fraction III by affinity chromatography on DNase I-Sepharose 4B (data not shown), suggesting that this protein is indeed actin. In our purification procedure, binding of actin to PC (step V) could be prevented by the addition of low levels of ATP (0.5 mM) to the binding buffer. Tubulin, which has been shown to co-purify with the ligase during PC chromatography (20), could be separated from the enzyme by the glycerol gradient centrifugation step (fraction IV). Purification of ligase by microtubule protein affinity chromatography as proposed by others (18) was not successful in our hands. We found that some tubulin was released from the matrix during elution of the enzyme even after having washed the affinity column extensively with high salt prior to use. Affinity columns using PC-tubulin gave similar results.

A major difficulty in purification is the loss in total enzyme activity occurring at the steps following the ATP-Sepharose column. This loss was overcome by addition of glycerol to the buffer. The stabilization effect of glycerol was also exploited in storage of the final enzyme preparations. In the presence of 40 or 20% glycerol the enzyme can be stored for at least 6 mo at -70°C at a protein concentration of ~20 μ g/ml without detectable loss in activity.

Purified ligase shows a sedimentation coefficient of 3.2 S when analyzed by glycerol gradient centrifugation (Fig. 2, A and B). This value corresponds to a molecular mass of about 43,000 for a globular protein (16). Taken together with the value of 40,000 seen in SDS-gel electrophoresis (Fig. 1) homogeneous ligase behaves as a globular protein containing a single polypeptide chain with a mass of ~40,000 daltons (see also reference 18). Since it has been proposed that ligase forms

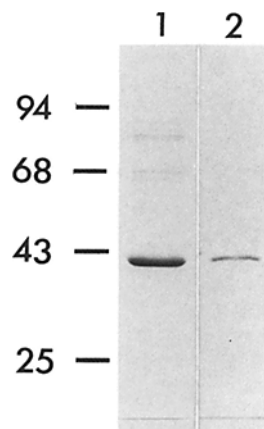


FIGURE 1 SDS PAGE of purified tubulin-tyrosine ligase. (Lane 1) Fraction V (PC column); (lane 2) fraction VI (tyrosine affinity column). About 4 and 1 μ g of enzyme protein, respectively, were electrophoresed on 10% SDS gels which were stained with Coomassie Blue.

a 1:1 complex with the tubulin dimer (23), we examined the sedimentation coefficient of the enzyme on a 10–20% glycerol gradient after preincubation with or without PC-tubulin. As shown in Fig. 2, *A* and *B*, the *S*-value of ligase preincubated with excess PC-tubulin was about 7.3 S, corresponding to a molecular weight of approximately 150,000. The kinetic constants of the purified ligase for different radioactively labeled amino acid substrates were essentially as described (18, 23, 24). The Michaelis constant for L-tyrosine as substrate was found to be 25.4 μM , for L-DOPA 186 μM , and for L-phenylalanine 432 μM . Tyramine was incorporated at very low levels. Because of the extremely low reaction velocity it was not possible to determine the K_M value.

Isolation of Monoclonal Antibodies to TTL

Because we used small amounts of ligase (between 10 and 20 μg of fraction V per injection) for immunization, we expected a poor antigenic response resulting in few ligase-positive clones. Thus, the initial screening procedure was designed to identify clones directed against the ligase whether the antibodies present were able to neutralize the enzyme activity or not.

For the first screening the original antigen (fraction V) was used in the peroxidase spot test. Supernatants from 38 out of 145 wells showing cell growth gave a positive reaction. A further analysis was made with an enzyme test which identified 4 of the 38 wells as positive. The second test (see Materials and Methods) was designed as a precipitation assay in which immobilized anti-mouse IgGs were incubated with supernatants to bind any secreted mouse antibody. After extensive washing the immobilized complex was mixed with partially

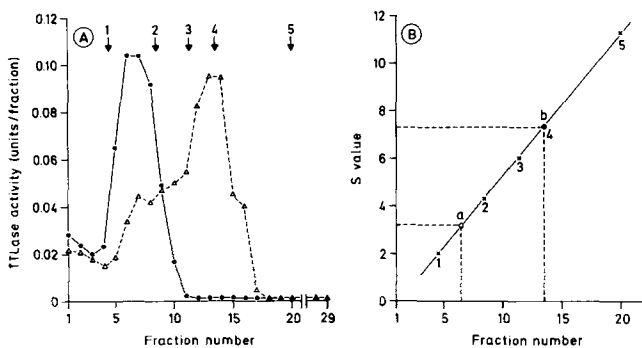


FIGURE 2 Sedimentation behavior of tubulin-tyrosine ligase. (A) a 400- μl sample of purified ligase (fraction V; 7.7 μg of enzyme protein) was loaded onto a linear glycerol gradient (10–20%, vol/vol) in a 25 mM K^+MES buffer (pH 6.8, supplemented with 1 mM MgCl_2 , 20 mM KCl, and 1 mM DTT) and centrifuged for 20 h at 4°C and 41,000 rpm (286,500 g) in a Beckman SW41 Ti rotor. After centrifugation, 0.45 ml-fractions were collected and a 10- μl aliquot of each fraction was assayed for TTL (●). Another sample of the ligase preparation (identical enzyme concentration) was preincubated in the presence of 0.9 mg/ml PC-tubulin for 15 min at 20°C. Gradient centrifugation and determination of enzyme activity (Δ) was performed as described above. The direction of sedimentation is from left to right. The following marker proteins were run in parallel tubes: 1, ribonuclease A; 2, bovine serum albumin; 3, tubulin dimer; 4, aldolase; 5, catalase. (B) A plot of the known *S*-values for the marker proteins (X) vs. fraction number. An almost straight line is provided by the marker proteins. The sedimentation coefficients of TTL (a) and the complex formed in the presence of tubulin (b) were estimated from the intersection points with the calibration curve as indicated (○).

purified ligase and the resulting supernatants were tested for enzymatic activity. Instead of measuring the incorporation of ^3H -labeled tyrosine, we took advantage of the previously described ability of the rat monoclonal α -tubulin antibody (clone YL 1/2) to distinguish between tyrosinated and detyrosinated α -tubulin (31, 32). Therefore carboxypeptidase A-treated microtubule protein was used as substrate. The reaction mixtures were spotted on to a nitrocellulose sheet and the normal peroxidase spot test was performed using YL 1/2 antibody (Fig. 3). In this test a negative result indicates that the immobilized antibody complex had removed the ligase from the solution to be tested thereafter in the enzyme assay. Thus the particular hybridoma supernatant giving this result should contain ligase antibody. The screening assay based on YL 1/2 antibody is more convenient than the conventional assay that measures the incorporation of radioactively labeled tyrosine.

The four independently isolated clones were termed LA/C4, LB/B5, LD/A3, and LE/C1. The clone LA/C4 was the best antibody producer when grown as ascites. All four monoclonal antibodies were identified as IgG1 by double immunodiffusion. The antibodies detected purified ligase on immunoblots (Fig. 4). However, when tested on the original pig brain extract no decoration was found suggesting that the enzyme is present in too low a quantity to be detected by the blotting technique used. This explanation was confirmed in experiments where a small amount of purified ligase was added to the brain extract before the sample was run on SDS gels (Fig. 4, lanes 3 and 6). None of the four murine monoclonal antibodies is able to inhibit ligase activity directly, when purified antibodies were directly added to the reaction mixture. Ligase, even when bound to the immobilized antibodies, stays enzymatically active (data not shown).

LA/C4 antibodies purified from ascites fluid were coupled to Sepharose. Brain and liver extracts from various species

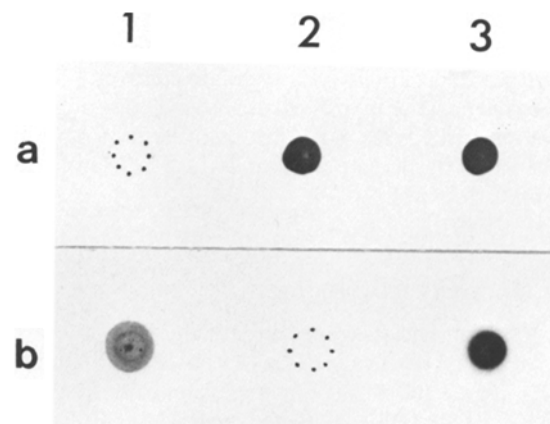


FIGURE 3 Screening assays to identify anti-ligase-positive hybridomas. (a) Test one: purified ligase (0.5 μl of fraction V) was spotted on to a nitrocellulose sheet which was subsequently saturated with BSA and incubated with supernatants from wells containing PA1 myeloma cells (1), LA/C4 (2), or LB/B5 cells (3), respectively, followed by peroxidase-conjugated rabbit anti-mouse IgGs and substrate. (b) Test two: different microtubule protein samples (1 μg) were spotted onto nitrocellulose, saturated with BSA and incubated with the rat monoclonal YL 1/2 antibody followed by peroxidase-conjugated rabbit anti-rat IgGs and substrate. (1) Unmodified microtubule protein, (2) CPA-treated microtubule protein, (3) CPA-treated microtubule protein re-aminoacylated by purified ligase with L-tyrosine (for details see Materials and Methods).

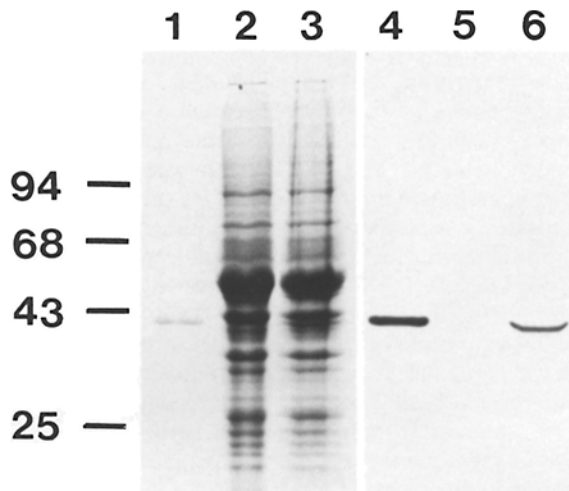


FIGURE 4 Immunoblot analysis of the monoclonal anti-ligase LA/C4 antibody. Lanes 1 and 4 represent $\sim 0.4 \mu\text{g}$ purified ligase (fraction V). Lanes 2, 3, and 5, 6 represent the total pig brain lysate. For lanes 3 and 6, $\sim 0.4 \mu\text{g}$ purified ligase was added to the cell lysate before running the gel. All lanes were 10% SDS gels. Lanes 1–3 were stained with Coomassie Blue, the corresponding lanes 4–6 are immunoblots labeled with $[^{125}\text{I}]\text{-LA/C4}$. Purified ligase (lane 4) is easily detected. The amount of enzyme present in the crude brain extract (lane 5) is too low to allow detection. Lane 6 serves as a control. Molecular weights, $\times 10^{-3}$.

were incubated with immobilized antibody. After extensive washing Sepharose beads were boiled in sample buffer and the supernatants analyzed by SDS-slab gels (Fig. 5) and immunoblotting using LA/C4 antibodies directly labeled with ^{125}I . LA/C4 antibodies detect ligase in liver and brain from pig (Fig. 5) and moreover from cow and rat but fail to react on the same tissues from mouse and chicken (data not shown). Thus the antibody shows a moderate cross species reactivity and does not distinguish between the enzyme present in liver and brain. Preliminary immunofluorescence microscopical experiments using various cultured cells have not revealed any significant labeling in comparison to controls. The failure to detect the enzyme by this procedure may be connected to the fixation procedures used. Alternatively it is more likely that the level of enzyme present is just too low to allow detection.

Purification of TTL by Immunoaffinity Chromatography

The starting material was essentially the same as described for conventional purification (see Materials and Methods and Table I). The supernatant obtained after the first tubulin polymerization step was incubated overnight at 4°C with LA/C4 immobilized on Sepharose. The material was filled into a column and extensively washed with buffer containing 1.5 M NaCl as described in Materials and Methods. Active ligase was eluted with buffer containing 3 M MgCl_2 . After removal of the MgCl_2 by dialysis against stabilization buffer containing 20% glycerol the enzyme can be stored at -70°C without detectable loss in activity. Ligase prepared in this manner showed an enzymatic activity comparable to that obtained in fraction V of Table I and had a purity of at least 95% when analyzed by SDS gels. Because of the formation of fibrous aggregates, presumably polymerized actin and tubulin, during the incubation with the affinity matrix it has been necessary

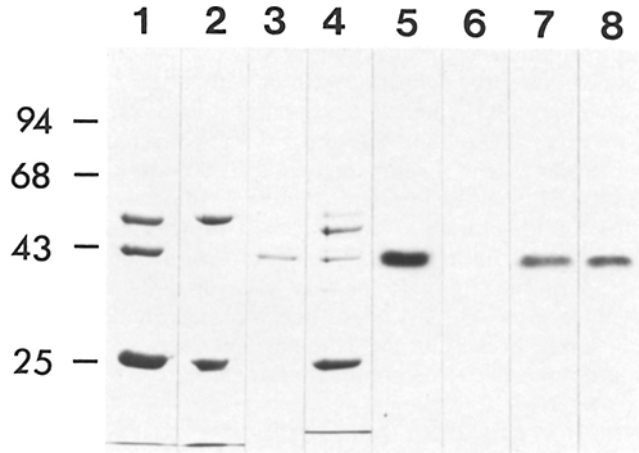


FIGURE 5 Analysis of immunoprecipitated and affinity-purified ligase. Lanes 1–4 represent a 10% SDS gel stained with Coomassie Blue and lanes 5–8 are the corresponding immunoblots using LA/C4 directly labeled with ^{125}I . Lanes 1, 2, 4, 5, 6, and 8 show the results on immobilized LA/C4 antibodies. Pig brain extracts (lanes 1, 2, and 5, 6) and pig liver extracts (lanes 4 and 8) were incubated with the affinity matrix. After extensive washing the matrix was stripped either directly with SDS sample buffer (lanes 1 and 4 and 5 and 8) or first with 3 M MgCl_2 followed by SDS sample buffer (lanes 2 and 6). Lanes 3 and 7 represent approximately $1 \mu\text{g}$ of pig brain ligase eluted from immobilized LA/C4 antibodies with 3 M MgCl_2 . Molecular weights, $\times 10^{-3}$.

to start the purification with the supernatant of the warm centrifugation step rather than using a very crude brain extract. The immunoaffinity purification provides ~ 1 to 1.2 mg of enzyme per kilogram of pig brain. This value corresponds approximately to the content of ligase estimated by enzymatic activity.

DISCUSSION

Using several conventional purification steps we have purified TTL from pig brain. The essentially homogeneous enzyme can be stored in the presence of glycerol for several months without loss in activity. This property allows a study of tubulin tyrosination-detyrosination since a pure and constantly active preparation is obtained. Our enzyme preparation is free of tubulin that has been shown to remain associated under conditions that separate microtubule-associated proteins from tubulin dimers (20). The ligase preparation shows essentially the same properties as found for a partially purified enzyme (23) and for a highly purified ligase (18). The K_M values for tyrosine, phenylalanine, and L-DOPA previously reported have also been confirmed (2, 23).

TTL is a globular monomeric protein containing a single polypeptide chain of molecular weight 40,000 (see also reference 18). This assignment is independently verified by immunological results (see below). On the basis of gel filtration experiments, a one to one complex between ligase and tubulin has been described (23), and this result is now confirmed for the pure enzyme by glycerol gradient centrifugation. The carboxyterminal tyrosine residue of α -tubulin displays a rapid *in vivo* turnover in cultured cells (27). Nevertheless the *in vitro* turnover number of the ligase is rather low. The most active enzyme preparation had a specific activity of only 762 U/mg from which a turnover number of about 30 molecules of tyrosine per molecule of enzyme per minute can be calculated.

Enzymatic assays for TTL that can first be done in the supernatant of the microtubule polymerization step indicate the presence of ~1.5 mg of ligase per kilogram of brain. Thus an extensive purification is required and the relative instability of enzyme raises several difficulties. Although we have found a stabilizing effect of glycerol a standard purification scheme as outlined in Table I provides only 0.14 mg of enzyme per kilogram of pig brain and requires a large number of time-consuming steps. These difficulties have now been overcome by a second purification scheme based on immunoaffinity chromatography using monoclonal antibodies to TTL. As documented above this fast and convenient procedure provides ~1–1.2 mg of enzyme per kilogram of pig brain and can be directly applied to the supernatant fraction obtained after microtubule assembly has occurred. Thus steps II to VI in the standard biochemical purification scheme are substituted by one affinity chromatography.

Probably due to the small amount of enzyme originally available we have only obtained a few monoclonal antibodies. Because these antibodies fail to inhibit the enzyme activity they have not been further explored in physiological experiments such as microinjection into cultured cells. Nevertheless these monoclonal antibodies were extremely suitable for enzyme purification. TTL remains bound to immobilized antibodies at 1.5 M NaCl and is released at 3 M MgCl₂. Thus the enzyme is not subjected to drastic and damaging solvents. The enzyme obtained is comparable to that resulting from the time-consuming standard preparation. The four monoclonal antibodies have a moderately broad cross-species reactivity among mammals and detect the enzyme also in non-neuronal tissue. Thus it is possible to isolate ligase from liver by immunoaffinity chromatography.

The physiological role of the reversible addition of a tyrosine to the carboxyterminal glutamate of α -tubulin remains unclear. It has been suggested that tubulin de-tyrosination/tyrosination influences subcellular compartmentation of tubulin (19; see also reference 27), membrane excitability of axons (27), interaction of tubulin with MAPs (10), and the nucleation/elongation process of microtubule formation (10). The preparation of an essentially homogeneous, stable TTL available in milligram quantities should now provide a good tool for biochemical experiments. A final assessment of the physiological significance of the tyrosine cycle may, however, depend on microinjection studies using antibodies which inhibit the enzymatic activity of TTL. Although we did not yet isolate such antibodies, the ease with which the ligase can now be prepared encourages further attempts.

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Note Added in Proof: Using the immunoaffinity purified TTL as antigen, we have recently succeeded in raising polyclonal antibodies

as well as a monoclonal antibody both of which inhibit the enzymatic activity of the ligase.

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