

Tubulin-Tyrosine Ligase Has a Binding Site on β -Tubulin: A Two-domain Structure of the Enzyme

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Abstract. Tubulin-tyrosine ligase and $\alpha\beta$ -tubulin form a tight complex which is conveniently monitored by glycerol gradient centrifugation. Using two distinct ligase monoclonal antibodies, several subunit-specific tubulin monoclonal antibodies, and chemical cross-linking, a ligase-binding site was identified on β -tubulin. This site is retained when the carboxy-terminal domains of both tubulin subunits are removed by subtilisin treatment. The ligase-tubulin complex is also formed when ligase is added to $\alpha\beta$ -tubulin carrying the monoclonal antibody YL 1/2 which binds only to the carboxyl end of tyrosinated α -tubulin. The β -tubulin-binding site described here explains the extreme substrate specificity of ligase, which does not act on other cellular proteins or carboxy-terminal peptides derived from detyrosinated α -tubulin. Differential

accessibility of this site in tubulin and in microtubules seems to explain why ligase acts preferentially on unpolymerized tubulin. Ligase exposed to V8-protease is converted to a nicked derivative. This is devoid of enzymatic activity but still forms the complex with tubulin. Gel electrophoresis documents both 30- and a 14-kD domains, each which is immunologically and biochemically distinct and seems to cover the entire molecule. The two domains interact tightly under physiological conditions. The 30-kD domain carries the binding sites for β -tubulin and ATP. The 14-kD domain can possibly form an additional part of the catalytic site as it harbors the epitope for the monoclonal antibody ID3 which inhibits enzymatic activity but not the formation of the ligase-tubulin complex.

ALTHOUGH encoded by the mRNA (38), the carboxy-terminal tyrosine of α -tubulin shows turnover in cells and tissues (1, 4, 5, 22-24). While the process of detyrosination by a putative carboxypeptidase is still poorly understood (2, 3, 17), certain aspects of the reverse reaction are well established in vitro (4, 5, 23-25). Tubulin-tyrosine ligase recharges the detyrosinated α -tubulin of $\alpha\beta$ -tubulin in an ATP-dependent reaction (5, 24). The enzyme is highly specific for α -tubulin and does not act on other proteins present in the cell or in crude extracts (1, 4, 23). The reaction seems to occur on soluble $\alpha\beta$ -tubulin rather than on microtubules (1, 23, 36) in line with an isolatable complex formed between ligase and $\alpha\beta$ -tubulin (20, 24, 28). Ligase activity has been found in various vertebrates (22, 34), in several invertebrates (10, 16), and even in protozoa (32). In addition, all but two of the many α -tubulin genes described to date predict the presence of a carboxy-terminal tyrosine residue (7, 14, 39). In agreement, the monoclonal antibody YL 1/2 (15), which recognizes only the carboxyl end of tyrosinated α -tubulin (40, 42), has a broad cross-species reactivity extending from yeast to man and higher plants (15, 40-42). Removal of the tyrosine abolishes antibody reactivity (40, 41). Various experiments including some with YL 1/2 show that the carboxy-terminal domains of α - and β -tubulin are not directly involved in the formation of the microtubular structure. It

rather seems that these domains extend from the filament wall into the cytoplasm (26, 29, 42). Thus, the physiological role of the tyrosine turnover of α -tubulin has remained undefined.

Previous work on the α -tubulin-specific ligase was greatly hampered by a cumbersome purification, yielding only small amounts of enzyme, which was found to be rather unstable (20). We have recently developed a rapid immunoaffinity purification (28). This procedure and the use of glycerol as a stabilizing agent has made it possible to obtain milligram quantities of ligase as a stable enzyme preparation. In the course of experiments aimed at the characterization of two distinct monoclonal antibodies to ligase, we found that the enzyme has two domains. Here we show that the stable complex between ligase and $\alpha\beta$ -tubulin involves a very strong binding site, which surprisingly locates to the β -tubulin subunit.

Materials and Methods

Materials

Chemicals were obtained as follows. L-[3,5- 3 H] Tyrosine (specific activity, 57 Ci/mmol), [α - 32 P]ATP (3,000 Ci/mmol), and sodium [125 I]iodide were from Amersham International, Amersham, UK; ATP and GTP were from Waldhof, Mannheim, Federal Republic of Germany; dimethylpimelimidate

dihydrochloride (DMP)¹ and the IODO-GEN reagent were from Pierce Chemical Co., Rockford, IL; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were from SERVA, Heidelberg, FRG. Subtilisin was from Sigma Chemical Co., St. Louis, MO. *Staphylococcus* V8-protease was from Boehringer Mannheim GmbH, Mannheim FRG. Phosphocellulose (P11) and Whatman 3MM filter paper were from Whatman Ltd., Maidstone, UK; CNBr-activated Sepharose 4B and DEAE-Sephacel were from Pharmacia, Freiburg, FRG. All other chemicals were from Sigma Chemical Co.

Peroxidase-conjugated second antibodies were obtained from DAKO-PATTS, Copenhagen, Denmark. The rat monoclonal α -tubulin antibodies (clones YL 1/2 and YOL 34) were generously provided by Dr. J. Kilmartin, Cambridge, England (15). The β -tubulin-specific mouse monoclonal antibody (6) (clone DMIB) was from Amersham International. Nitrocellulose membrane filters (BA 83, 0.2 μ m) were from Schleicher & Schuell, Dassel, FRG.

Methods

Pig brain microtubule protein was isolated by three cycles of temperature-dependent assembly/disassembly in 0.1 M Pipes, pH 6.5, 1 mM MgSO₄, 1 mM EGTA, 1 mM GTP, and 1 mM 2-mercaptoethanol (30). The first polymerization was done in the presence of 4 M glycerol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Homogeneous tubulin (PC-tubulin) was prepared from microtubule protein by phosphocellulose (P11) chromatography (31). Proteins were stored in aliquots at -70°C . Tubulin-tyrosine ligase from pig brain was purified by immunoaffinity chromatography using a mouse monoclonal antibody specific for ligase (clone LA/C4) as described (28). After elution from the affinity matrix with 3 M MgCl₂, the enzyme was dialyzed overnight at 4°C against stabilization buffer (25 mM K⁺MES [pH 6.8], 0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol [DTT], 20% glycerol [vol/vol]) and then stored in aliquots at -70°C . Before use all protein samples were centrifuged for 20 min at 4°C in a Beckman TL 100 centrifuge at 50,000 rpm.

SDS PAGE and protein transfer from gels to nitrocellulose was performed as previously described (37). Usually peroxidase-labeled second antibodies were used for visualization. Protein concentrations were determined by the Lowry reagents using BSA as standard (18). Purified rabbit anti-ligase IgG's were directly iodinated using the IODO-GEN method (9).

Antibody Production

Monoclonal Antibodies. Four 6-wk-old female BALB/c mice were immunized at 3-wk intervals with affinity-purified ligase (100–200 μ g enzyme/injection) using Freund's complete adjuvant for the first injection and incomplete adjuvant for the two subsequent injections. Sera were tested by immunodiffusion. The spleen cells from the mouse giving the strongest reaction were fused with cells from the myeloma line PAI (33) as described (28). Colony supernatants were screened by ELISA using 96-well microtiter plates. All incubation steps were done at 37°C . Approximately 0.5 μ g purified ligase was provided per well. After 2 h, the wells were treated with PBS-BSA (4% wt/vol in PBS) for 2 h and then incubated with the colony supernatants. After a 2-h incubation, wells were washed five times with PBS before addition of peroxidase-conjugated rabbit anti-mouse IgG's (diluted 1:500 into PBS-BSA). After 1 h, wells were extensively washed with PBS, and then the substrate (H₂O₂; *o*-phenylene diamine) was added. Positive supernatants were further tested in the ligase enzyme assay to identify antibodies which inhibited enzymatic activity. In addition, Western blotting was done. Positive colonies were cloned twice by limiting dilution. Ascites fluids were produced in BALB/c mice. After precipitation with 50% ammonium sulfate, dialysis against 10 mM sodium phosphate, pH 7.4, and loading on to DEAE-Sephacel, IgG's were eluted with 40 or 100 mM sodium phosphate, pH 7.4.

Polyclonal Antibodies. Two rabbits were immunized with affinity-purified ligase (200–400 μ g enzyme/injection) using standard immunization procedures. Anti-ligase antibodies were isolated by affinity chromatography on ligase coupled to CNBr-activated Sepharose 4B. Specific antibodies were eluted with 0.2 M sodium acetate, pH 2.5.

Fab Fragments. IgG's purified from ascites fluids (clones LA/C4 and ID3) were digested for 8 h at 37°C in 0.1 M sodium citrate, pH 4.0, with 10 μ g pepsin per mg IgG (21). The reaction was stopped by addition of 1 M Tris-HCl, pH 8.8, to bring the pH to 7.0. F(ab)₂ fragments were reduced with cysteine and then alkylated with iodoacetamide as described (21). Gel electrophoresis indicated full conversion of IgG to Fab.

1. *Abbreviation used in this paper:* DMP, dimethylpimelimidate dihydrochloride.

Preparation of Cross-linked Antibody Matrix. ID3 antibodies were coupled to CNBr-activated Sepharose 4B (~ 3 mg of purified IgG per ml of settled gel) and then cross-linked with 20 mM DMP essentially as described by Schneider et al. (27).

Ligase Assay. Enzyme reactions were in 50 μ l containing 25 mM K⁺morpholinoethanesulfonic acid (MES) (pH 6.8), 150 mM KCl, 12.5 mM MgCl₂, 2.5 mM ATP, 1 mM DTT, 100–200 μ g microtubule protein or PC-tubulin, 0.1 mM L-[³H]tyrosine, and 10 μ l enzyme solution containing maximally 0.05 U as defined previously (23). For antibody tests, IgG's or Fab fragments were preincubated with ligase for 20 min at 4°C before adding the substrate.

Photoaffinity Labeling. Direct photoaffinity labeling of intact and V8-treated ligase by ATP was performed essentially as described for myosin and actin (19). Between 10 and 50 μ g protein in 50 μ l ligase stabilization buffer, 10 μ Ci of [α -³²P]ATP, and 1 μ M unlabeled ATP were irradiated at 4°C for 20 min by UV light (wavelength 254 nm) at a distance of 4–6 cm. Samples were directly analyzed by SDS PAGE. Gels were stained for protein with Coomassie R-250 dye, dried, and autoradiographed using Fuji RX-film.

Protease Treatment. Ligase (0.5 mg/ml in stabilization buffer) was digested with *Staphylococcus* V8-protease (5 μ g/ml) for 1 to 8 h at 37°C . Incubation for 3 h was sufficient to cleave >90% of the ligase into two defined fragments (see Results). V8-protease was removed by affinity chromatography on purified rabbit anti-V8-protease IgG's (11) covalently coupled to Sepharose 4B (3 mg of purified IgG per ml of settled gel) and equilibrated with ligase stabilization buffer. The V8-protease-depleted digest was stored in aliquots at -70°C .

Glycerol Gradient Centrifugation. Usually 300 μ l samples were loaded onto linear glycerol gradients (10–20% [vol/vol]) in 25 mM K⁺MES buffer (pH 6.8, supplemented with 1 mM MgCl₂, 20 mM KCl, and 1 mM DTT) and centrifuged for 20 h at 4°C at 41,000 rpm in an SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA). 0.4-ml fractions were collected and a 10- μ l aliquot of each fraction was assayed for enzyme activity. In parallel, a 1- μ l aliquot of each fraction was spotted onto nitrocellulose sheets (13). Ligase was detected by autoradiography after exposure of the sheet to [¹²⁵I]-labeled rabbit anti-ligase IgG's.

Cross-linking Studies. (a) Dimethylpimelimidate dihydrochloride (DMP): Microtubule protein or PC-tubulin (1 mg/ml) and intact or V8-digested ligase (20–100 μ g/ml) were dialyzed against the cross-linking buffer (0.1 M triethanolamine, pH 8.2, 20 mM KCl, 2 mM MgCl₂, 2 mM DTT, 10% glycerol [vol/vol]) for 2 h at 4°C . A 5 mg/ml stock solution of DMP was prepared in cross-linking buffer and the pH was readjusted to 8.2 with 1 N NaOH. 150 μ l of the stock solution was added per ml protein solution. Reaction was for 10 or 30 min at room temperature. The extent of cross-linking was estimated by SDS PAGE and Western blots.

Addition of ATP (1 mM) did not affect the degree of cross-linking. Specific cross-linking was still obtained when the pH was lowered to 7.6 or 6.8 in order to minimize a possible denaturation of tubulin. Due to the reduced reactivity of amino groups especially at pH 6.8 the extent of cross-linking was smaller than at pH 8.2.

(b) 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC): PC-tubulin (1 mg/ml) and ligase (0.1 mg/ml) in polymerization buffer (0.1 M Pipes, pH 6.5, 1 mM MgSO₄, 1 mM EGTA, 1 mM GTP, 1 mM 2-mercaptoethanol) were incubated for 1 h at 4°C . EDC was added from a stock solution (100 mM in polymerization buffer) to a final concentration of 10 mM. After 10 or 30 min at room temperature, reaction products were analyzed as above.

Subtilisin Digestion of Tubulin. Subtilisin was dissolved at 1 mg/ml in water and stored in aliquots at -70°C . Digestion of PC-tubulin (5 mg/ml) was performed as described (26) in polymerization buffer at 37°C using 50 μ g subtilisin/ml. The reaction was stopped by adding 1% by volume of 1% (wt/vol) PMSF in DMSO.

Polymerization Studies. Microtubule protein was mixed with ligase at different concentrations in polymerization buffer and kept for 20 min on ice. After temperature shift to 37°C , assembly was monitored by turbidity at 350 nm. After 30 min at 37°C , aliquots were negatively stained with 2% uranylacetate on carbon-coated grids and examined by electron microscopy.

Results

Two Distinct Monoclonal Antibodies: ID3 Inhibits Enzymatic Activity of Ligase

Monoclonal antibody LA/C4 allows a rapid purification of ligase as the native enzyme is released from the immunoaffinity matrix by 3 M MgCl₂ (28). As LA/C4 does not in-

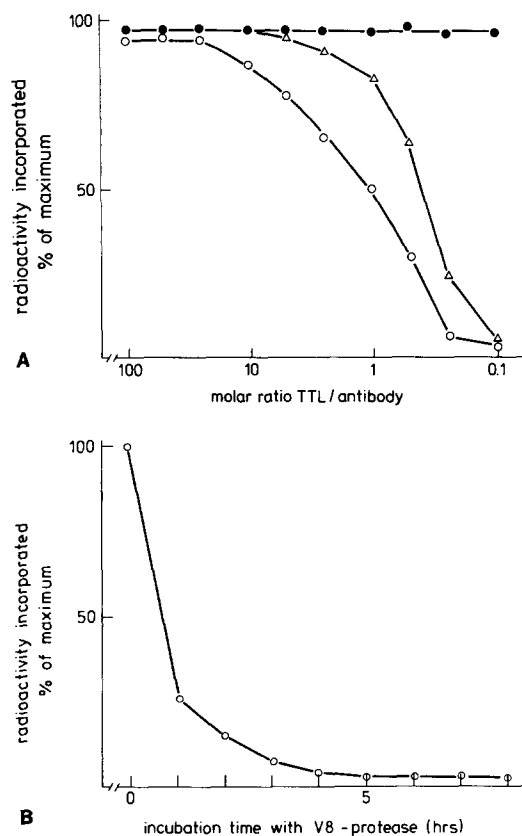


Figure 1. (A) Inhibition of ligase activity by monoclonal antibodies. 1 μ g of enzyme was preincubated with different concentrations of purified ID3 and LA/C4 IgG's or Fab fragments before addition of the tubulin substrate. LA/C4 IgG's (solid circles); ID3 IgG's (open circles); and ID3 Fab-fragments (open triangles). (B) Loss of ligase activity by V8-protease treatment. Ligase was incubated with V8-protease at 37°C; aliquots were removed each hour and incubated at 4°C for 20 min with rabbit anti-V8-protease IgG's coupled to Sepharose to remove the protease. After sedimentation of the Sepharose 10- μ l aliquots of all samples were assayed for ligase activity. Note the loss of ligase activity after prolonged protease treatment.

hibit enzymatic activity (Fig. 1 A), a new set of monoclonal antibodies was raised. Antibody ID3 was particularly interesting. This IgG₁ recognizes ligase specifically in Western Blots (Fig. 2) and effectively inhibits tyrosine incorporation into tubulin. This property is fully retained in Fab fragments prepared by standard procedures (Fig. 1 A). The properties of the two monoclonal antibodies and their ligase epitopes are summarized in Table I. In the course of establishing these properties several interesting aspects of the ligase-tubulin interaction emerged.

Two-domain Structure of Ligase

Purified ligase treated with V8-protease shows a characteristic time course of digestion when analyzed by SDS PAGE. Under standard conditions the 40-kD protein is slowly converted into two fragments, which have apparent molecular masses of 30 and 14 kD, respectively (Fig. 2). Western blotting showed that the two fragments are immunologically distinct. ID3 recognized only the 14-kD fragment, while LA/C4 was specific for the 30-kD fragment (Fig. 2). Prolonged treatment with protease showed further cleavage of

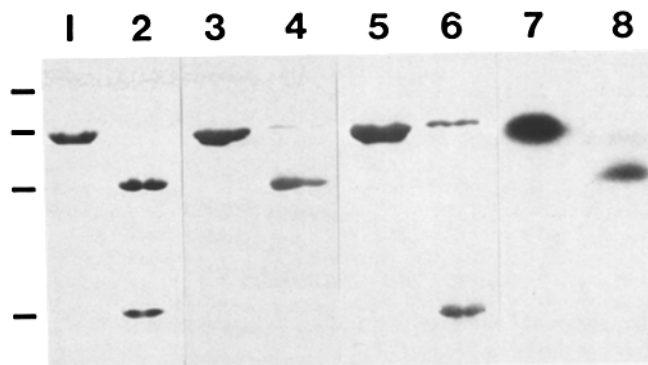


Figure 2. Analysis of ligase and its V8-protease digestion products by SDS PAGE (lanes 1 and 2) and corresponding immunoblots (lanes 3-6); localization of the ATP-binding site (lanes 7 and 8). Intact ligase (lane 1) was incubated with V8-protease for 3 h at 37°C and the digest was passed through a column of immobilized rabbit anti-V8-protease IgG's to remove the protease (lane 2). Corresponding gel slices of intact and fragmented ligase were transferred to nitrocellulose and incubated with LA/C4 (lanes 3 and 4) or ID3 IgG's (lanes 5 and 6). Bound antibodies were visualized with anti-mouse peroxidase-conjugated antibodies. Of the two ligase-specific antibodies, LA/C4 labels the 30-kD fragment (lane 4) and ID3 the 14-kD fragment (lane 6). Note that some uncleaved ligase is still present in the digest (lanes 4 and 6; see text). For the autoradiograms in lanes 7 and 8, intact ligase (lane 7) and its V8 digest (lane 8) were incubated with [α -³²P]ATP, irradiated by UV light, and analyzed by SDS PAGE as described in Materials and Methods. Note the label on ligase and the 30-kD fragment. Molecular mass standards (66, 46, 29, and 14 kD) are indicated by bars at the left side (top to bottom).

the fragments (not shown). Thus, in most of the following experiments preparations were used that still contained ~10% uncleaved ligase (note for instance lanes 4 and 6 in Fig. 2). Separate experiments showed that the activity of ligase is lost parallel to the incubation with V8-protease (Fig. 1 B).

As in several attempts we were unable to separate the two fragments by gel filtration, we considered the possibility that the domains interact even after the protease has introduced at least one polypeptide break in a putative hinge region. Fragmented as well as intact ligase were tightly bound by ID3 antibodies immobilized on Sepharose 4B. Neither intact enzyme nor its fragments were released when the salt was raised to 1 M NaCl. However, when the column was washed at 37°C with 4 M urea solution, the 30-kD fragment eluted while intact ligase and the 14-kD fragment remained bound

Table I. Properties of Ligase Monoclonal Antibodies

	LA/C4	ID3
Antibody type	IgG ₁	IgG ₁
Elution of antigen from antibody	3 M MgCl ₂	urea > 4 M (37°C)
Inhibition of ligase activity	-	+
Inhibition of tubulin-ligase complex formation	-	-
Epitope	30 kD	14 kD

Table II. Properties of the Two Ligase Domains Defined by V8-Protease

	30 kD	14 kD
Monoclonal antibody	LA/C4	ID3
ATP site	+	-
β -Tubulin-binding site	+	-

(not shown). This result agrees with the Western blots showing that the epitope of ID3 is located on the 14-kD fragment (see above and Fig. 2, lane 6). In addition the experiments show that the two ligase domains still interact until 4 M urea is used. Additional support for this view is given below from experiments involving glycerol gradient centrifugation.

The two domains of the ligase differ not only immunologically but also functionally. Intact ligase and the digest were incubated with [α - 32 P]-labeled ATP and subjected to UV photolysis as described for other nucleotide-triphosphate binding proteins (19). Subsequent gel electrophoresis and autoradiography showed ATP cross-linked to ligase and its 30-kD fragment, while the 14-kD domain remained free of label (Fig. 2, lanes 7 and 8). The currently established properties of the two domains are summarized in Table II.

ID3 Antibody Inhibits Enzymatic Activity but Does Not Interfere with the Formation of the Ligase-Tubulin Complex

Ligase is known to form a one-to-one complex with $\alpha\beta$ -tubulin which can be monitored by glycerol gradient centrifugation and has an S value of 7.3 (28). Complex formation between ligase and tubulin was studied with both ligase antibodies. To avoid possible problems of steric hindrance, Fab fragments rather than intact IgG molecules were used. Complex formation was followed by gradient centrifugation and enzyme activity assays of the resulting fractions. Alternatively ligase was detected by dot assay using [125 I]-labeled rabbit anti-ligase antibodies. This assay is very reproducible and easy to perform. It is particularly useful for those ligase complexes which have lost enzymatic activity due to the binding of ID3 antibodies.

Ligase and the ligase-tubulin complex sedimented with S values of 3.2 and 7.3, respectively (28). Both species were clearly separated by glycerol gradient centrifugation (Fig. 3 A; see also reference 28) in contrast to free tubulin with an S value of 6 and the ligase-tubulin complex (see inset in Fig. 3 A). In addition to the position of ligase and the ligase-tubulin complex, Fig. 3 A also shows the complexes formed between Fab fragments of LA/C4 and the free enzyme or the enzyme-tubulin complex. These antibody carrying complexes retain enzymatic activity (see also Fig. 1 A). Thus, ligase and its complexes are detected both by enzymatic activity and the dot assay. Only the latter procedure could be used to follow the complexes formed between Fab fragments of ID3 antibody and the ligase. Fig. 3 B shows that the ligase-Fab complexes of ID3 sediment at the same position as seen for LA/C4 in Fig. 3 A. Preincubation with tubulin shifts the complex with ID3 to the position also recognized with LA/C4 (compare A and B in Fig. 3). Thus, the inhibiting antibody ID3 still allows the formation of the ligase-tubulin complex. The inhibition of enzymatic activity by ID3 (Fig. 1)

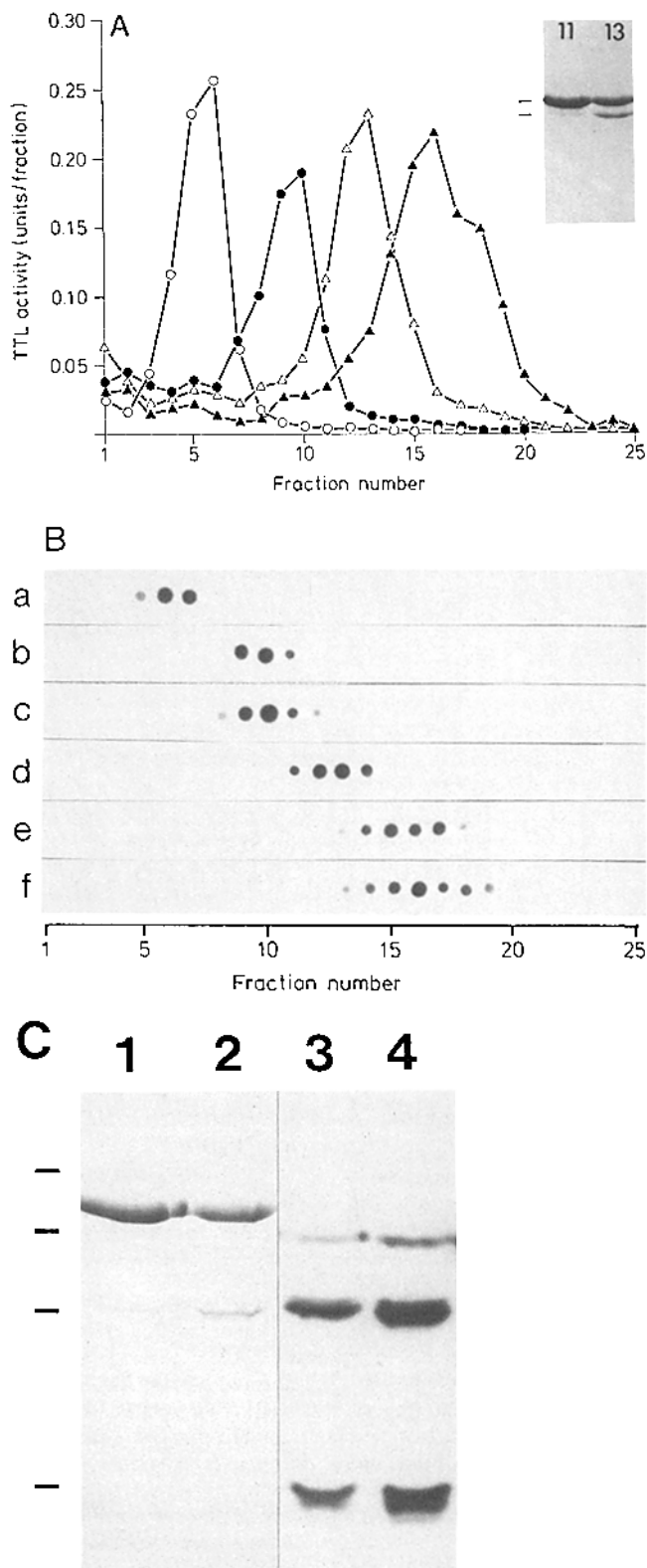


Figure 3. Sedimentation behavior of ligase and its complexes. 300- μ l samples containing various combinations of intact ligase (25 μ g) or V8-treated ligase (10 μ g), PC-tubulin (500 μ g), and Fab fragments (300 μ g) of LA/C4 and ID3 antibodies were preincubated for 20 min at room temperature before being loaded onto linear glycerol gradients (10–20% [vol/vol]). After centrifugation, 0.4-ml fractions were collected. (A) 10- μ l aliquots of all fractions were assayed for ligase activity (TTL activity). The direction of sedimentation is from left to right. Gradients shown are as follows: ligase

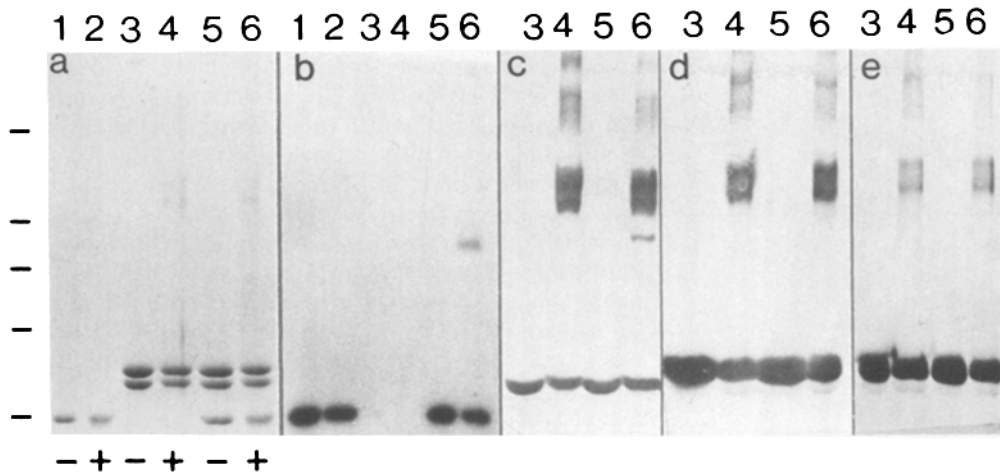


Figure 4. Immunological analysis of a complex between ligase and tubulin obtained by chemical cross-linking. (a) Ligase (lanes 1 and 2), PC-tubulin (lanes 3 and 4), and PC-tubulin plus ligase (lanes 5 and 6) were incubated with (+) or without (-) DMP as described in Materials and Methods, and analyzed by SDS PAGE on a 6% slab gel. (b) An identical gel as in a was transferred to nitrocellulose and incubated with [¹²⁵I]rabbit anti-ligase antibodies before being autoradiographed. Note the presence of a 100-kD ligase-positive band obtained

by cross-linking in the presence but not absence of tubulin. This band, only poorly seen by dye staining, is clearly detected by ligase antibodies. (c-e) Gel slices corresponding to lanes 3-6 in a were transferred to nitrocellulose sheets which were incubated with different monoclonal tubulin antibodies. Bound antibodies were visualized with peroxidase-conjugated second antibodies. (c) Anti- β -tubulin; (d) anti- α -tubulin (clone YL 1/2); (e) anti- α -tubulin (clone YOL 34). Note that the 100-kD ligase-tubulin cross-link is only detected by anti- β -tubulin antibodies (c, lane 6) and not by anti- α -tubulin antibodies (d and e). Molecular mass standards indicated at the left are as follows: 205, 116, 92, 66, and 46 kD (top to bottom).

is therefore not due to an antibody or Fab molecule hindering the interaction of ligase with the tubulin substrate.

Glycerol gradient centrifugation was also used on V8-protease-treated ligase. The digested enzyme still formed a complex with $\alpha\beta$ -tubulin which by SDS gel electrophoresis and Western blots revealed both ligase fragments (Fig. 3 C).

Characterization of a Ligase-binding Site on β -Tubulin

To understand the $\alpha\beta$ -tubulin-ligase complex in more detail

(open circles); ligase and LA/C4 Fab fragments (solid circles); ligase and PC-tubulin (open triangles); ligase, PC-tubulin, and LA/C4 Fab fragments (solid triangles). Inset shows fractions 11 and 13 obtained from the ligase plus tubulin glycerol gradient (open triangles) analyzed on a 15% slab gel; bars indicate positions of tubulin subunits not resolved and ligase, respectively. (B) 1- μ l aliquots of each fraction of several glycerol gradients were spotted onto nitrocellulose sheets which were subsequently saturated with BSA and incubated with [¹²⁵I]rabbit anti-ligase antibodies before autoradiography. (a) Ligase; (b) ligase and LA/C4 Fab fragments; (c) ligase and ID3 Fab fragments; (d) ligase and PC-tubulin; (e) as for d but with LA/C4 Fab fragments as well; (f) as in d but with ID3 Fab fragments as well. Note the same shift of ligase (a) by both types of Fab fragments (b and c). Note also that the ligase-tubulin complex (c) is shifted by both Fab fragments (e and f) to the same extent. The positions of the various complexes seen in B are also found in A by enzyme tests as LA/C4 fragments do not inhibit the activity. (C) Fractions 11 (lane 1) and 13 (lane 2) obtained from a tubulin-ligase glycerol gradient which was run with V8-treated ligase instead of intact ligase, were analyzed by SDS PAGE on a 15% slab gel (for comparison see inset in A). An identical gel slice was transferred to nitrocellulose and incubated with [¹²⁵I]rabbit anti-ligase before being autoradiographed (lanes 3 and 4). Note that in addition to some uncleaved ligase present in the digest, V8-treated ligase still forms a complex with tubulin which contains both ligase fragments. Molecular mass standards indicated at the left are 66, 46, 29, and 14 kD (top to bottom).

we performed various cross-linking studies with DMP in solution. Complexes were analyzed by gel electrophoresis followed by immunoblotting. Purified ligase is a monomer (20, 28) and therefore no additional cross-linked species were found when ligase alone was treated with the reagent. Cross-linking of $\alpha\beta$ -tubulin alone was relatively low (Fig. 4). Incubation of the cross-linker with a mixture of both proteins yielded an additional band at \sim 100 kD. While this band was barely visible on the dye-stained gel (Fig. 4 a, see also Fig. 6), it was clearly recognized by polyclonal anti-ligase antibodies on the corresponding blots (Fig. 4 b, lane 6). Only two bands were recognized by the polyclonal and the two monoclonal ligase antibodies: the normal ligase at 40 kD and a tubulin-ligase complex at 100 kD. Treatment of the same blots with several monoclonal antibodies to tubulin (Fig. 4, c-e) showed that the 100-kD species was recognized only by the β -tubulin-specific antibody (clone DM1B) (Fig. 4 c) and not by two α -tubulin-specific antibodies (clones YOL34 and YL 1/2) (Fig. 4, d and e). Carbodiimide when used instead of DMP for cross-linking gave the same results. The resulting 100-kD complex of ligase was again only recognized by the β -tubulin-specific antibody and not by the α -tubulin-specific antibodies (not shown).

When V8-treated ligase was used instead of intact enzyme, the cross-linked ligase-positive species was shifted from 100 to \sim 85 kD (Fig. 5). This suggested that the complex was formed between the 30-kD fragment of ligase and β -tubulin. Immunoblotting confirmed this assumption. The cross-linked species was only detected with LA/C4 but not with ID3 antibodies (compare lanes 8 and 10 in Fig. 5).

Mild digestion of tubulin with subtilisin is supposed to remove \sim 30 carboxy-terminal residues from both α - and β -tubulin (26, 29). The actual cleavage sites on both tubulins have so far not been determined. The resulting derivative shows tubulin polymerization independent of microtubule-associated proteins (26, 29). In our hands, a proteolytic treatment of 30 min at 37°C provided a useful tubulin derivative. Immunoblots with YL 1/2 antibody, which is specific for tyrosi-

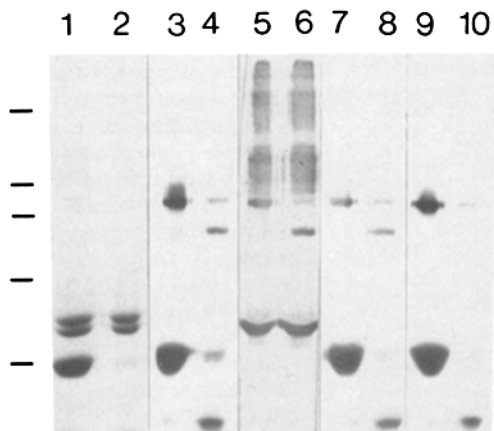


Figure 5. Cross-linking of V8-fragmented ligase with tubulin. PC-tubulin and intact ligase (lane 1) and PC-tubulin plus V8-treated ligase (lane 2) were incubated with DMP as described in Materials and Methods and analyzed by SDS PAGE on a 7.5% slab gel. V8-treated ligase ran close to the gel front and therefore barely detected by Coomassie staining (lane 2). Identical gel slices as in lanes 1 and 2 were transferred to nitrocellulose sheets, which were incubated with the following antibodies: [¹²⁵I]rabbit anti-ligase (lanes 3 and 4); β-tubulin antibody (lanes 5 and 6); LA/C4 antibody (lanes 7 and 8); ID3 antibody (lanes 9 and 10). Bound antibodies were visualized by autoradiography (lanes 3 and 4) or peroxidase-conjugated second antibodies (lanes 5–10). Molecular mass standards indicated at the left are as follows: 205, 116, 92, 66, and 46 kD (top to bottom). Note the formation of an 85-kD cross-linked species in the V8-digest positive for ligase with polyclonal antibodies (lane 4), monoclonal anti-ligase LA/C4 (lane 8), but negative with monoclonal anti-ligase ID3 (lane 10). This 85-kD species reacts with β-tubulin antibody (lane 6). Due to the presence of some unfragmented ligase, an additional band at 100 kD can be seen which is also decorated by ID3. Other slots provide controls with intact ligase rather than fragmented ligase.

nated α-tubulin (40, 42), gave no reaction indicating that the carboxyl end of α-tubulin had been removed.

Glycerol gradient centrifugation showed that subtilisin-treated tubulin retains binding of ligase (not shown). After cross-linking with DMP, two bands at 100 and 90 kD and the normal 40-kD enzyme were recognized by ligase-specific antibodies on corresponding blots (Fig. 6). The 100-kD band disappeared in preparations which had been more extensively treated with subtilisin (compare lanes 5 and 6 in Fig. 6). Immunoblots showed that the tubulin–ligase cross-link was reactive both with ligase (Fig. 6, lanes 4–6) and with β-tubulin-specific antibodies (Fig. 6, lanes 7–9).

We also used glycerol gradient centrifugation to see whether the monoclonal YL 1/2 antibody (15), which specifically binds to the carboxyl end of tyrosinated α-tubulin (40, 42), might interfere with the formation of the ligase–tubulin complex. Fig. 7 shows that upon preincubation with YL 1/2 antibody, the ligase–tubulin complex sedimented faster than the normal ligase–tubulin complex. Since in a normal preparation only some 10% of the tubulin is tyrosinated, most of the ligase–tubulin complex remained in its original position. These results show that the strong interaction between αβ-tubulin and ligase is not due to a binding site at the carboxyl end of α-tubulin, where the ligase acts enzymatically, but rather arises from a binding site on the β-tubulin.

Ligase Acts Preferentially on Tubulin

Several studies indicate that soluble tubulin molecules rather than microtubules are the preferred substrate of the ligase (1, 23). Thus conditions which enhance microtubule formation seem to reduce tyrosine incorporation (36). This view is in line with our experiments using taxol. When microtubular protein was preincubated with 10 μM taxol, tyrosine incorporation decreased to 20% of the control value in the standard assay. When ligase was mixed with taxol-stabilized microtubules, subsequent glycerol gradient centrifugation showed that essentially no enzyme sedimented with microtubules (not shown). These results suggest that the ligase-binding site on tubulin is changed upon transition from the tubulin monomer to the microtubular polymer. Thus we followed microtubule polymerization in the presence of increasing amounts of ligase using turbidity measurements (Fig. 8). Microtubule protein (0.5 mg/ml) was mixed in the cold with ligase (final concentration range, 0.05 to 1 mg/ml) and polymerization was started by temperature shift to 37°C. After 30 min at 37°C aliquots of all samples were also analyzed by negative staining in the electron microscope (not shown). Only at higher ligase concentrations was polymerization affected and at 0.3 mg/ml of ligase, complete inhibition was achieved (Fig. 8, curve *d*), no microtubules could be detected by electron microscopy.

Discussion

V8-protease was found to be a useful tool to define two distinct domains of tubulin–tyrosine ligase. These separate in

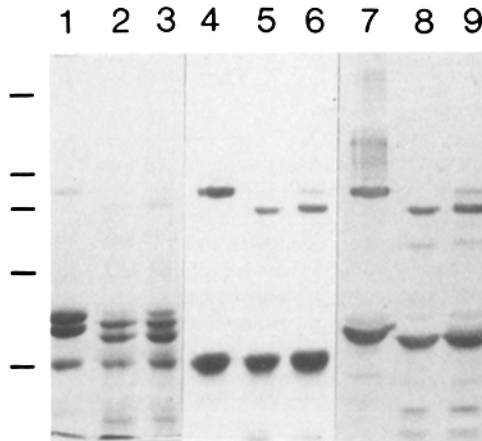


Figure 6. Analysis of ligase cross-linked to subtilisin-cleaved tubulin. Tubulin was treated with subtilisin for different times. The resulting digests were incubated with ligase and cross-linking was performed with DMP as described in Materials and Methods. Ligase cross-linked to normal tubulin (lane 1), 30-min tubulin digest (lane 2), and 20-min tubulin digest (lane 3) was analyzed by SDS PAGE on a 7.5% slab gel. Identical gel slices as lanes 1–3 were transferred to nitrocellulose sheets, which were incubated with [¹²⁵I]rabbit anti-ligase antibodies (lanes 4–6) or anti-β-tubulin antibody (lanes 7–9). Bound antibodies were visualized by autoradiography (lanes 4–6) or peroxidase-conjugated second antibodies (lanes 7–9). Molecular mass standards indicated at the left are as follows: 205, 116, 92, 66, and 46 kD (top to bottom). Note that all the cross-linked species displaying reactivity with ligase antibodies (lanes 4 to 6) are also recognized in lanes 7–9 by β-tubulin antibody.

SDS gels as 14- and 30-kD fragments, although they bind tightly to each other under native conditions. This binding was retained in 1 M salt, and treatment with 4 M urea at 37°C was necessary to separate the fragments. Of the two distinct monoclonal antibodies to ligase characterized here, ID3 was specific for the 14-kD fragment while LA/C4 only reacted with the 30-kD fragment. Thus it seems that the two fragments span different parts of the ligase molecule. As the molecular mass of ligase is ~43 kD (20, 28), the two fragments seem to cover the entire molecule. We cannot exclude, however, the possibility that a small peptide not detected by our current criteria was additionally released upon V8 treatment. The results indicate that the two domains of the ligase still tightly interact after the protease has introduced at least one polypeptide break into a connecting hinge region. For convenience the V8-treated enzyme is called nicked ligase.

Although nicked ligase is devoid of enzymatic activity it retains the ability to form the one to one enzyme-substrate complex with tubulin, which can be monitored by glycerol gradient centrifugation. The stability of this complex in solution allowed us to study ligase-tubulin interaction by two chemical cross-linking reagents. Complexes revealed by SDS PAGE were characterized by different monoclonal antibodies to tubulins and ligase. The results document an unexpected ligase-binding site on β -tubulin which involves the 30-kD ligase domain. As ligase catalyzes the tyrosination of the α -subunit, it has at least two contact points on its $\alpha\beta$ -tubulin substrate. One obviously involves the carboxyl end of α -tubulin where the enzyme acts, while the other lies within the β -tubulin. To assess the relative strength of the two sites in complex formation, we have made use of a tubulin derivative known to have lost the carboxy-terminal 3-kD domains on both subunits (26, 29). Subtilisin-treated $\alpha\beta$ -tubulin still interacted both with normal and nicked ligase. These results establish two important points on ligase-tubulin interaction. First, stable complex formation does not

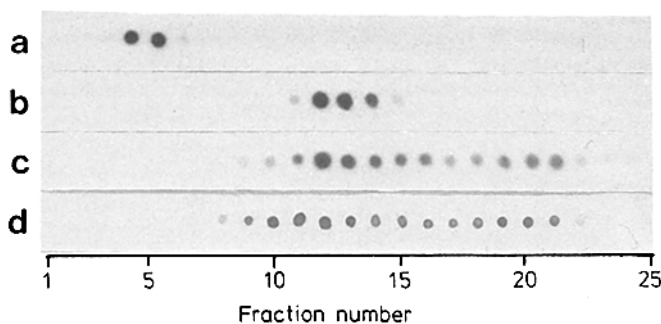


Figure 7. Sedimentation behavior of the tubulin-ligase complex in the presence of YL 1/2 antibody. 300- μ l samples containing ligase (25 μ g), PC-tubulin (500 μ g), and YL 1/2 IgG's (1 mg) were preincubated for 20 min at room temperature before being loaded onto linear glycerol gradients (10–20% [vol/vol]). After centrifugation, 0.4-ml fractions were collected and 1- μ l aliquots of all fractions of several glycerol gradients were spotted onto nitrocellulose sheets which were subsequently saturated with BSA. (a) Ligase; (b) ligase and PC-tubulin; (c and d) as in b but with YL 1/2 IgG's as well. a–c were incubated with [¹²⁵I]rabbit anti-ligase antibodies before autoradiography. YL 1/2 IgG's in d were visualized with peroxidase-conjugated second antibodies. The direction of sedimentation is from left to right. Note the shift of the tubulin ligase complex (b) by YL 1/2 (c and d).

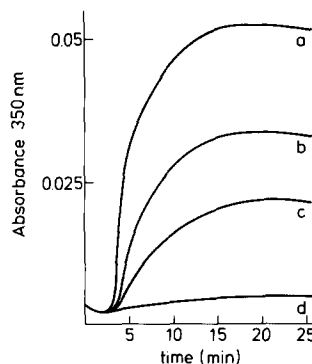


Figure 8. Polymerization of microtubule protein in the presence of ligase. Microtubule protein (0.5 mg/ml) and ligase (0.05–1 mg/ml) in polymerization buffer were preincubated for 20 min on ice before polymerization was initiated by shifting the temperature to 37°C. Ligase concentrations: (a) no ligase, (b) 0.1 mg/ml, (c) 0.2 mg/ml, (d) 0.3 mg/ml. Note complete inhibition of polymerization in curve d.

require the carboxyl end of α -tubulin, which is the site of enzymatic action. Second, the binding site on β -tubulin is outside its carboxy-terminal 3-kD domain. The first conclusion was also obtained on normal $\alpha\beta$ -tubulin without resorting to the subtilisin derivative. Antibody YL 1/2 binds to the carboxyl end of α -tubulin provided the tyrosine is present (40, 42). Gradient centrifugation documented a complex of $\alpha\beta$ -tubulin plus ligase carrying in addition the bulky IgG on the carboxyl end of the α -subunit. Thus, we conclude that the in vitro interaction between $\alpha\beta$ -tubulin and ligase observed by various techniques does not require the carboxyl end of α -tubulin which is used during catalysis.

The presence of a ligase-binding site on β -tubulin opens an explanation for the high specificity of the enzyme. It does not act in vitro on peptides spanning the carboxy-terminal residues of detyrosinated α -tubulin (24; our own unpublished results) or on denatured $\alpha\beta$ -tubulin (23); and in vitro and in vivo studies suggest that $\alpha\beta$ -tubulin is the only substrate in the cell (1, 4, 23). This high degree of enzymatic specificity is expected if the binding to β -tubulin is the prerequisite to enzymatic action at the carboxyl end of the α -tubulin present in the same $\alpha\beta$ -tubulin molecule. Future experiments have to explore whether the different tubulin isotypes have an influence on the extent of tyrosination of the α -tubulins, since only some 50% of total brain tubulin has been reported to be tyrosinable in vitro (1). Therefore, especially with respect to the heterogenous β -tubulins, a subclass of tubulin isotypes might not act as substrate for ligase (for a review on isotypes see references 7 and 39).

While there is general agreement that monomeric $\alpha\beta$ -tubulin rather than the microtubule is the preferred ligase in vitro substrate (1, 23, 36), the reason for this distinction was unclear as the carboxy-terminal domain of α -tubulin seems to protrude from the filament wall (26, 29, 42). This problem seems now overcome by the identification of a site on β -tubulin necessary for ligase binding. This site seems changed, weakened, or even hidden upon transition of tubulin to microtubules. This would explain why preparations of microtubular protein subjected to repeated cycles of polymerization and depolymerization lose most of the "contaminating" ligase activity (24; our unpublished results). The enzyme stays in the supernatant as tubulin-ligase complex once microtubules are harvested by centrifugation. Nevertheless, we found some ligase activity even in recycled microtubules. One possibility to account for this observation would be that tubulin molecules present at one or both ends of a microtu-

bule could still bind ligase possibly acting as a "capping" protein. Our preliminary results support this view. At least under in vitro conditions, noticeable inhibition of tubulin polymerization is found at high ligase concentration when microtubule assembly is monitored by turbidity measurements or electron microscopy.

Exploring the two domains of ligase delineated by V8-protease (Table II), we found that the ATP-binding site of the enzyme as well as its interaction site with β -tubulin are located on the 30-kD fragment. While we cannot yet attach a direct biochemical function to the 14-kD fragment, we note that it binds the monoclonal antibody ID3. This assignment is important as ID3 inhibits the enzymatic activity of ligase without interfering with the formation of the tubulin-ligase complex. The same properties (i.e., ability of complex formation but no enzymatic activity) are typical for nicked ligase. Thus the 14-kD fragment could carry part of the catalytic site. Even if the ATP-binding pocket locates to the 30-kD fragment as seen by photolysis experiments, the 14-kD domain could still be involved in recognizing the carboxyl end of α -tubulin. Alternatively the smaller domain may have an indirect influence to transduce the influence of a contact between ligase and β -tubulin on the catalytic center recognizing the carboxyl end of α -tubulin.

Although our experiments have unraveled several novel properties of tubulin-ligase interaction and offer an explanation for the high specificity of the enzyme, they don't define the physiological importance of the metabolic turnover of the carboxy-terminal tyrosine of α -tubulin. This is in part due to the poor characterization of the second process, which removes the tyrosine. This activity is usually called a carboxypeptidase (2, 3, 17). Its specificity for $\alpha\beta$ -tubulin predicts that in addition to recognizing the carboxy-terminal end of tyrosinated α -tubulin, it will have like the ligase an additional binding site on the $\alpha\beta$ -tubulin molecule. Several in vivo and in vitro experiments indicate that loss of the tyrosine occurs on microtubules rather than on the pool of soluble tubulin (17, 35). Whether the in vivo detyrosination is solely the function of a tubulin-specific carboxypeptidase rather than a reflection of a yet unidentified microtubule-mediated event is currently not known. Recent reports based on immunocytochemical studies open several questions as they invoke separate entities of microtubules harboring or lacking the carboxy-terminal tyrosine as in some cultured cell lines (12). In addition, during axonal maturation of the cerebellar cortex, axonal and dendritic microtubules seem to differ in their degree of tyrosination (8). It is tempting to speculate that such heterogeneous populations of microtubules might have distinct functions.

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