# Communication

## Tubulin Subunit Carboxyl Termini Determine Polymerization Efficiency*

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Cleavage of tubulin by subtilisin removes a small ($M_r$ < 2000) fragment from the C-terminal end of both $\alpha$ and $\beta$ subunits. The resulting protein is much reduced in negative charge. The cleaved, less acidic protein retains its competence to polymerize in a GTP-dependent and cold-, GDP-, and podophyllotoxin-sensitive manner and assembles into sheets or bundles of twisted filaments. The critical concentration for polymerization of the cleaved protein is about 50-fold lower than that for intact tubulin. It is proposed that the C termini of the subunits normally impede polymerization.

Tubulin is a heterodimer whose two subunits ($\alpha$ and $\beta$) have been sequenced, are composed of about 450 amino acids each, contain no internal cross-links, and have a molecular mass of very nearly 50,000 daltons (1, 2). Sequence alignment reveals 42% sequence homology between the two chains (55% if conservative substitutions are counted) suggesting a common evolutionary origin (3). Both subunits are acidic proteins, but the $\beta$ subunit is slightly more acidic than $\alpha$ (apparent pI = 5.3 ($\beta$), 5.4 ($\alpha$) (4)). The protein is subject to several post-translational modifications, such as phosphorylation of the $\beta$ and tyrosination of the $\alpha$ subunit (5, 6). Both of these modifications occur at or near the carboxyl terminus of their respective subunit. These modifications do not affect the ability of the protein to polymerize *in vitro*, and their exact role *in vivo* is not know, though tyrosination may be involved in the partition of tubulin into membranes (7).

The C-terminal end of both subunits is remarkably rich in acidic amino acids, especially glutamic acid (1, 2). At physiological pH, this sequence will be highly charged and probably in an extended conformation (8). If so, it should be more accessible than the folded region of the protein to attack by enzymes, including but not limited to those involved in the post-translational modifications mentioned.

Proteolytic digestion can be a useful tool to study protein structure and function as well as a regulatory mechanism of protein function *in vivo* (as *e.g.* the formation of fibrin polymers (8, 9)). In previous studies, partial proteolysis has been used as a probe of tubulin structure (10) and function (11). These studies indicated that certain proteases would selectively cleave the $\alpha$ or $\beta$ subunit into two unequal large frag-

ments, which apparently remain together, retaining more or less native characteristics. Tubulin so cleaved was still competent to assemble, given certain buffer conditions. However, in other buffers, assembly competence was lost, and cleaved tubulin acted as a substoichiometric poison of assembly of intact tubulin. As an approach to understanding the regulatory role of the C terminus of tubulin subunits, we have investigated the properties of tubulin, which has been cleaved by subtilisin, since we have found that this protease cleaves off the C terminus of both subunits.

### MATERIALS AND METHODS

Tubulin was prepared by phosphocellulose purification of rat brain microtubule protein, prepared by two cycles of temperature-dependent polymerization, as described (12) and stored at −70 °C following drop freezing in liquid nitrogen. Subtilisin BPN was obtained from Sigma, dissolved at 1 mg/ml in water, frozen in aliquots, and stored at −70 °C. Aliquots were thawed once only.

Digestion of tubulin was performed with subtilisin at 1:100 (w/w) in tubulin assembly buffer (0.1 M MES,[1] 1 mM MgCl$_2$, 1 mM EGTA, pH 7.0), in the presence of 1 mM GTP at 30 °C. The reaction was terminated by addition of 1% by volume of 1% (w/v) phenylmethanesulfonyl fluoride in dimethyl sulfoxide.

Assembly was monitored by turbidity at 350 nm using a thermostated Cary Model 219 spectrophotometer. Following digestion and addition of phenylmethanesulfonyl fluoride, samples were placed on ice. Assembly was initiated by transferring the sample from ice to the prewarmed (37 °C) cuvette holder.

Sodium dodecyl sulfate electrophoresis was performed in gel slabs using a modification of the Laemmli method (13). Gels contained 9% acrylamide and 0.6% Acrylaide (FMC) and were cast on Gelbond PAG (FMC). The lower gel buffer pH was 9.2, and SDS in the electrode buffer (0.1%) was lauryl sulfate (Sigma) or equivalent (14). Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250, destained, and air-dried (15). The migration of tubulin is anomalous in these gels, but this is useful as its results in excellent separation of the $\alpha$ and $\beta$ subunits.

Agarose electrophoresis was performed in 1% agarose gels (electrophoresis grade, Bethesda Research Laboratories) prepared in assembly buffer, and cast on Gelbond (FMC). Samples in assembly buffer were mixed 1:1 with 20% glycerol in assembly buffer containing 0.002% Bromphenol Blue and kept on ice until loading on the gel. Gels were run at 5–10 °C submerged in assembly buffer, which was recirculated during electrophoresis. The gels were stained and destained as the SDS gels and air dried.

### RESULTS AND DISCUSSION

When tubulin is exposed to subtilisin at 30 °C for various times and the products are analyzed by SDS-gel electrophoresis, it is seen that the enzyme does not extensively degrade the protein (Fig. 1A). Rather, the size of both subunits is only slightly reduced. When the time course of proteolysis is monitored by nondenaturing agarose electrophoresis (Fig. 1B), it is seen that tubulin is initially converted to a few slower migrating species. As digestion continues, it accumulates in a single slowly migrating band. SDS-gel electrophoresis of protein digested to this point reveals a virtually complete loss of the $\alpha$ and $\beta$ bands, which are replaced by bands of slightly lower molecular weight (Fig. 1A, *lane c*). The two main bands are referred to as $\alpha'$ and $\beta'$. Digestion for an hour longer does

[1] The abbreviations used are: MES, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid; SDS, sodium dodecyl sulfate; PIPES, 1,4-piperazinediethanesulfonic acid.
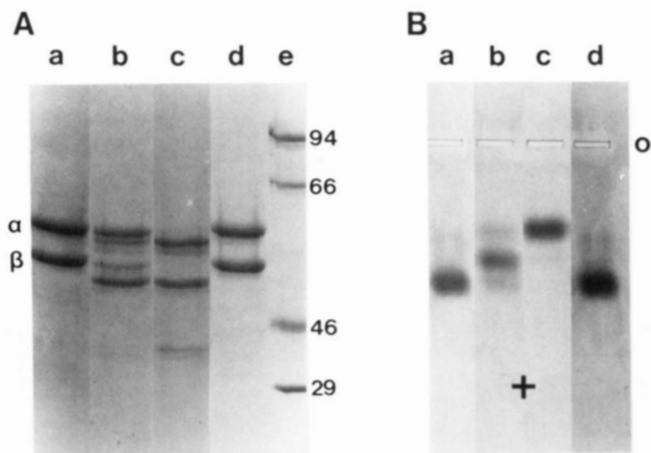
**A**

a b c d e



**B**

a b c d



FIG. 1. **Electrophoresis of intact and subtilisin-cleaved tubulin.** Tubulin (2 mg/ml) was digested with subtilisin at 30 °C and prepared for electrophoresis as described under "Materials and Methods." "Intact tubulin" was treated exactly the same except that addition of subtilisin was omitted. A, sodium dodecyl sulfate-gel electrophoresis. *Lanes a* and *d*, intact tubulin; *b*, 5-min digestion; *c*, 40-min digestion; *e*, molecular weight standards: phosphorylase (96,000), bovine serum albumin (66,000), ovalbumin (46,000), carbonic anhydrase (29,000). *B*, agarose electrophoresis. *o*, origin. Samples are the same as in *A*.

TABLE I

*Charge properties of intact tubulin and a hypothetical C-terminal cleaved tubulin*

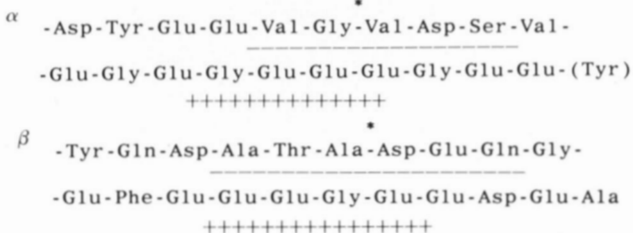| | Intact | | Cleaved* | |
|---|---|---|---|---|
| | $\alpha$ | $\beta$ | $\alpha'$ | $\beta'$ |
| | *no. amino acid residues* | | | |
| Acidic | 64 | 61 | 56 | 51 |
| Basic | 54 | 48 | 54 | 48 |
| Excess acidic | 10 | 13 | 2 | 3 |

• Cleaved indicates C-terminal 15 residues removed (see sequence below and text).

C-Terminal Sequence of Tubulin Subunits
\*, hypothetical cleavage site for example above.
– – – –, proposed region of subtilisin cleavage (based on reduced apparent molecular weight).
++++, proposed region of subtilisin cleavage (based on reduced apparent charge).

$\alpha$    -Asp-Tyr-Glu-Glu-Val-Gly-Val-Asp-Ser-Val-

    -Glu-Gly-Glu-Gly-Glu-Glu-Glu-Gly-Glu-Glu-(Tyr)
    ++++++++++++++

$\beta$    -Tyr-Gln-Asp-Ala-Thr-Ala-Asp-Glu-Gln-Gly-

    -Glu-Phe-Glu-Glu-Glu-Gly-Glu-Glu-Asp-Glu-Ala
    ++++++++++++++++

Sequence shown is porcine brain (1, 2). Rat brain $\alpha$ is identical.

not substantially alter this pattern (results not shown). On these gels, the *apparent* molecular weight (mean ± S.D., *n* = 9) of the $\alpha'$ subunit is 58,200 ± 700 (compared to 60,300 ± 500 for $\alpha$) and that of $\beta'$ is 49,500 ± 1,200 (compared to 52,500 ± 1,100 for $\beta$). The true molecular weight difference between the cleaved and intact protein is indeterminate, since the difference in migration of the intact subunit proteins is not due to a difference in molecular weight (16). Nonetheless, it is clear that the cleaved proteins are similar in size to the uncleaved proteins.

This small reduction in size is accompanied by a large

reduction in charge, as shown by agarose gel electrophoresis (Fig. 1*B*). The ratio of the anodal mobility of the cleaved to the intact protein is 0.6. Since the agarose matrix is nonsieving for proteins, even an order of magnitude larger than tubulin (17), this reduction in mobility implies a loss of negative charge. From the known sequence of tubulin (1, 2) and the small reduction in size, the large reduction in negative charge can only be explained by a C-terminal cleavage. By comparison, cleavage of 15 amino acids from the N terminus would remove only one acidic residue and two basic residues, resulting in an increase in net negative charge.

The data in Fig. 1 suggest a small discrepancy in the locus of the cleavage site in the C terminal between estimates made by reduction in apparent molecular weight (Fig. 1*A*) and charge (Fig. 1*B*). The former suggests a size reduction of ~15 amino acid residues, and a hypothetical case for such a cleavage is given in Table I. Such cleavage would yield an 80% reduction in excess negative charge whereas in Fig. 1*B* we observe only a 40% reduction amounting to a loss of 9 acidic residues. Assuming an approximately equal contribution of loss of charge in each subunit (both $\alpha$ and $\beta$ appear to be reduced in size), then the sites of cleavage will be approximately 7 residues from the C termini (see Table I). The present data do not permit a definitive choice between these two extremes and we conclude that subtilisin cleaves tubulin subunits at a site between 7 and 15 amino acids from the C terminus.

Following digestion, the protein can still polymerize (Fig. 2). When digestion is performed as described under "Materials and Methods," assembly is initiated following a short lag period. This assembly is reversed by cold, or by addition of 5 mM GDP or 50 $\mu$M podophyllotoxin (*curve a*). In the absence of GTP, subtilisin digestion results in a modest increase in absorbance, but assembly is not initiated unless GTP is added to the system (*curve b*). This assembly, like that under standard conditions (*curve a*), is reversible by cold treatment. Thus specific assembly and not simple aggregation is being monitored. The uncleaved pure tubulin will not assemble at these conditions and concentration. However, at higher protein concentrations the intact protein will assemble, and its assem-
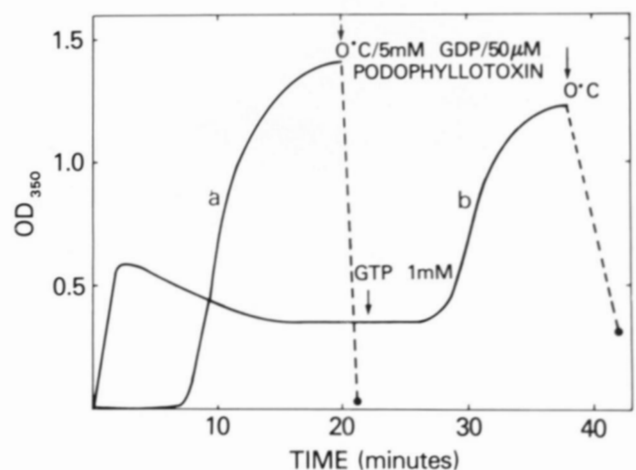


FIG. 2. **Polymerization of subtilisin-digested tubulin.** Tubulin, at 1.5 mg/ml, was incubated at 37 °C in assembly buffer + 2 mM GTP. Intact tubulin showed no increase in absorbance after 1 h under these conditions. *Curve a*, digestion was as described under "Materials and Methods" and at the time indicated by the *arrow*, the cuvette was cooled to 0 °C, or GDP was added to 5 mM, or podophyllotoxin was added to 50 $\mu$M. *Curve b*, GTP was omitted from the initial mix, subtilisin was added at time 0, and GTP was added at the indicated time.
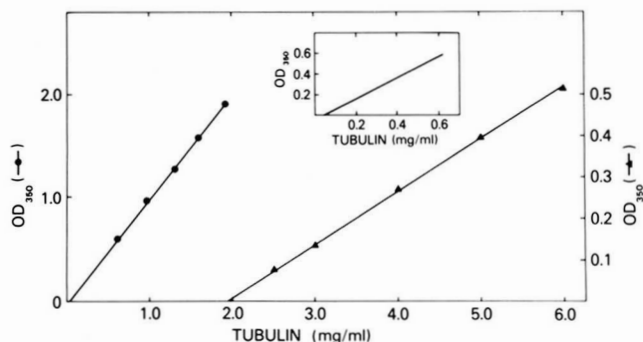
FIG. 3. **Critical concentrations for polymerization of intact (▲——▲) and cleaved (●——●) tubulin.** Tubulin was digested with subtilisin at 30 °C for 45 min and the reaction was terminated with phenylmethanesulfonyl fluoride and ice. Assembly was induced by warming to 37 °C at the protein concentrations indicated. Plateau absorbance values are plotted. The *inset* depicts the extrapolated low concentration range for subtilisin-cleaved tubulin.
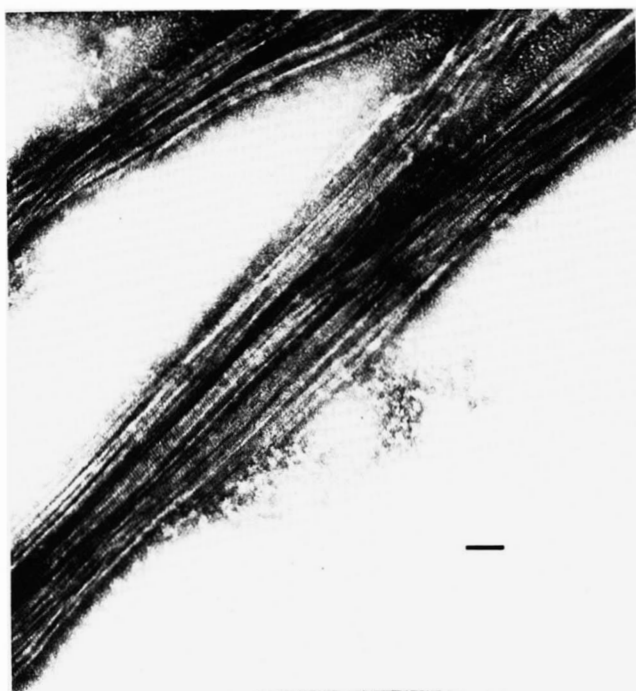


FIG. 4. **Electron microscopy of polymer.** Subtilisin-digested tubulin was polymerized at 37 °C; a drop was placed on a Formvar-coated grid and stained with 1% uranyl acetate. Magnification approximately × 195,000. *Bar*, 50 nm.

bly is GTP-dependent and cold-, GDP, and podophyllotoxin-sensitive (18).

The ability of cleaved tubulin to polymerize, under conditions in which the intact protein could not, suggested the possibility that a downward shift has occurred in the critical concentration (defined as the concentration below which no polymerization occurs). The pure tubulin used here will not polymerize at the concentrations of these experiments (1–1.5 mg/ml), as this is below its critical concentration (≥2 mg/ml) (Fig. 3). On the other hand, subtilisin cleavage of the C terminus results in a marked lowering of the critical concentration by between 1 and 2 orders of magnitude. As shown in Fig. 3, *inset*, the data extrapolate to a critical concentration of ~0.04 mg/ml. Above the critical concentration, polymer formed is a linear function of total concentration of subtilisin-tubulin, as is the case for tubulin above *its* critical concentration (Fig. 3).

The polymerization of cleaved tubulin results in a greater turbidity than expected for the total protein present. This suggests that the polymer, though specific and sharing characteristics of formation with microtubules (GTP-requiring, cold-, GDP-, and drug-sensitive), is in fact a form different from microtubules. Electron microscopy of negatively stained samples shows that this is the case (Fig. 4). The cleaved protein forms ordered arrays of filaments which are different from microtubules in that they are not closed and show clear cross-striations. They resemble the sheets and twisted filament structures formed from tubulin in high concentrations of glutamate or PIPES buffer (19). The effect of different conditions of polymerization on the form of the polymer produced from the cleaved protein is currently being examined.

Thus the principal results of limited subtilisin cleavage of tubulin are a substantial reduction of the net charge on the protein, most probably at the C-terminal end, and a highly significant reduction in the critical concentration for assembly. A reduction in critical concentration of intact tubulin can be effected by addition of microtubule-associated proteins. This may be due, in part, to neutralization of the charge on the C terminus. A clear example of this type of effect is the induction of polymerization by organic polycations (20). Charge neutralization at this locus may also explain the ability of elevated concentrations of inorganic divalent cations to induce polymerization (18). Our results are consistent with an interpretation wherein the intact C terminus would impede polymerization due to charge-charge repulsion between dimers. High concentrations of protein are required to overcome this barrier and drive the polymerization reaction unless this charge is reduced by some regulatory agent (such as microtubule-associated proteins) or action (such as C-terminal cleavage).

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