Effect of specific proteolytic cleavages on tubulin polymer formation

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The capacity for self-polymerization and shape of the tubulin polymers assembled after digestion with trypsin, Pronase, chymotrypsin, subtilisin, *Staphylococcus aureus* proteinase V8 and proteinase K were investigated. Digestion with trypsin, Pronase or chymotrypsin resulted in a decrease in the ability of tubulin for self-assembly, whereas limited proteolysis with subtilisin, *S. aureus* proteinase V8 or proteinase K resulted in an increase in such ability. The shape of the assembled polymers varied from typical microtubules (after the treatment with trypsin or Pronase) to sheets (after the treatment with chymotrypsin) and from hooked microtubules with a constant polarity (after the treatment with subtilisin) to the disappearance of a defined polarity of such polymers (after the treatment with *S. aureus* V8 proteinase or proteinase K). These results indicate that the tubulin C-terminal regions are involved in the regulation of microtubule polymerization, shape, directional growth and lateral interactions between tubulin protofilaments.

INTRODUCTION

The most characteristic feature of tubulin is its capacity for self-assembly from the promoter to a closed polymeric structure with a defined polarity (Amos, 1979). Limited proteolysis has been used to analyse microtubule protein structure and function (Love *et al.*, 1981; Brown & Erickson, 1983; Serrano *et al.*, 1984a, 1986; Mandelkow *et al.*, 1985; Serrano & Avila, 1985; Sackett *et al.*, 1985). Recently it was shown that the removal of a C-terminal fragment of both tubulin subunits by subtilisin digestion increased their capacity for self-assembly (Serrano *et al.*, 1984a; Sackett *et al.*, 1985). However, limited proteolysis with other proteinases (Brown & Erickson, 1983; Maccioni & Seeds, 1983; Serrano *et al.*, 1984b) resulted in the inhibition of this process.

After limited proteolysis, the assembled tubulin polymer could be different from that of microtubules. For example, the assembly of subtilisin-digested tubulin results in the appearance of hooked microtubules (Serrano *et al.*, 1984a), showing a constant polarity for the hooks, as in the case of cellular microtubules (Heidemann & McIntosh, 1980). This polarity has been related to the directional growth of the polymer (Heidemann & McIntosh, 1980).

Our objectives in the present study were (a) to analyse the regions involved in regulation of the initiation of tubulin assembly, and (b) to analyse the regions involved in determining the correct morphology, polarity of microtubule polymer and lateral interactions between polymers.

Our results indicate the involvement of specific regions of the tubulin molecule in facilitating the initiation of microtubule assembly and in determining the morphogenetic features of the assembled tubulin polymers.

MATERIALS AND METHODS

Purification of tubulin

Tubulin from pig brain was prepared by temperature-dependent cycles of assembly–disassembly by following the procedure of Shelanski *et al.* (1973), and was stored

as pellets at -70°C . Immediately before use, pellets were resuspended in buffer A (0.5 mM-MgCl₂/2.0 mM-EGTA/Mes/NaOH buffer, pH 6.4) and a third cycle of assembly–disassembly was performed. Tubulin depleted of microtubule-associated proteins was obtained by phosphocellulose chromatography as described by Weingarten *et al.* (1975). Characterization of phosphocellulose-purified tubulin by gel electrophoresis showed no other bands apart from tubulin subunits.

Proteolytic digestion

Tubulin in buffer A at a concentration of 2 mg/ml was used routinely in all experiments. Proteinases were resuspended in buffer A at 0°C at a concentration of 1 mg/ml, separated into 20 μl fractions and stored in liquid N₂. Controlled proteolysis of tubulin was performed by incubation of tubulin with the different proteinases, as indicated in Table 1.

After digestion, 1 mM-phenylmethanesulphonyl fluoride was added and the samples were either subjected to electrophoresis after being mixed with electrophoresis sample buffer as indicated by Laemmli (1970) or subjected to polymerization–depolymerization cycles. In the latter case, digested tubulin samples were incubated for 15 min at 37°C in buffer A containing 1 mM-GTP with or without 10% (v/v) dimethyl sulphoxide. Polymerized protein was fractionated from the unpolymerized protein by centrifugation for 3 min in a Beckman Airfuge at 25°C . The polymerized protein was resuspended in buffer A at 0°C , incubated at 0°C for 15 min and centrifuged again for 2 min in the Airfuge to remove insoluble aggregates. The supernatant of this centrifugation was incubated as indicated above to perform another polymerization cycle. The polymerized protein was isolated by centrifugation for 5 min in a Beckman Airfuge at room temperature. Protein content was quantified by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Mapping of the proteolytic fragments

Peptide maps of digested α - or β -tubulin subunits were constructed after a further formic acid cleavage of

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Table 1. Conditions for controlled proteolysis of tubulin

Proteinase	Supplier	Enzyme/ tubulin ratio (% w/w)	Time (min)	Temperature (°C)
Subtilisin (Carlsberg)	Sigma	1	30	30
Trypsin	Sigma	1.25	30	30
Pronase	Calbiochem	0.25	30	30
Chymotrypsin	Sigma, Boehringer	1.25	30	30
<i>Staphylococcus aureus</i> V8 proteinase	Miles	2	30	30
Proteinase K	Merck	0.1	30	30

Asp-Pro bonds, as described by Sonderegger *et al.* (1982) and as indicated previously (Serrano *et al.*, 1984a).

Electrophoresis

Proteinase-digested tubulin samples were adjusted to 1% (w/v) SDS/1 M-glycerol/2% (v/v) 2-mercapto-ethanol/0.001% Bromophenol Blue, boiled for 3 min and analysed by electrophoresis in SDS/polyacrylamide slab gels by following the procedure of Laemmli (1970), after which the gels were stained with Coomassie Blue (Fairbanks *et al.*, 1971). In some cases a previous step of

carboxymethylation (Crestfield *et al.*, 1963) was performed to obtain better separation of α - and β -tubulin subunits.

Electron microscopy and optical diffraction

Digested or undigested polymerized tubulin samples (2 mg/ml) were centrifuged in a Beckman Airfuge at 4 °C for 2 min and then incubated in buffer A containing 1 mM-GTP and 10% (v/v) dimethyl sulphoxide for 10 min at 37 °C in order to polymerize tubulin (Himes *et al.*, 1977). The samples (50 μ l) were then layered over a sucrose cushion (100 μ l) [30% (w/v) in buffer A containing 1 mM-GTP] and centrifuged in a Beckman Airfuge for 7 min at 25 °C. The supernatant was removed and the pellet fixed by incubation with 1% (v/v) glutaraldehyde in buffer A at 25 °C for 15 min. The pellet was washed with buffer A, post-fixed in 1% (w/v) OsO₄ in buffer A and block-stained with 2% (w/v) uranyl acetate in 70% (v/v) ethanol during dehydration through a graded series of acetone. The fixed samples were then embedded in Vestopal W resin, and ultrathin sections were cut with a glass knife, at a cutting angle of 4 °C, in a Sorvall MT2B ultramicrotome. Sections were stained with 1% (w/v) lead citrate and viewed in a Jeol 100B electron microscope.

For optical diffraction, polymerized, digested or undigested tubulin (2 mg/ml) was fixed with 1% (v/v) glutaraldehyde for 10 min at 25 °C, diluted 1/500 in buffer A at 25 °C, and centrifuged on to carbon/collodion grids in a Beckman Airfuge (5 min at 25 °C). The grids were stained with 1% (w/v) uranyl acetate, and micrographs of the polymers were obtained. Optical diffraction of the micrographs was performed as previously described (Serrano *et al.*, 1984a).

RESULTS

Effect of limited proteolysis of tubulin on its capacity for self-assembly

Limited proteolysis of tubulin can result in either the enhancement or the inhibition of its self-assembly, depending on the proteinase used (Brown & Erickson, 1983; Maccioni & Seeds, 1983; Serrano *et al.*, 1984a,b). Whereas trypsin or chymotrypsin digestion decreases the capacity of tubulin to polymerize (Maccioni & Seeds, 1983; Serrano *et al.*, 1984b), subtilisin digestion increases tubulin polymerization capacity (Serrano *et al.*, 1984b; Sackett *et al.*, 1985). In the latter case, a cleavage near the C-terminus of both tubulin subunits was produced by

Fig. 1. Proteolytic digestion of tubulin

Tubulin (2 mg/ml) was digested with subtilisin (S), proteinase K (PK) or *S. aureus* V8 proteinase (V8) as indicated in the Materials and methods section for 30 min at 30 °C. The digestion was stopped after 5 min of incubation by boiling the samples in electrophoresis buffer. The samples were subjected to electrophoresis in a 5–15% polyacrylamide slab gel. Undigested tubulin (Pc) was used as control. Tubulin subunits (α and β) lacking a small fragment proximal to the terminal ends of α - or β -subunits (α' and β' respectively) are indicated.

Table 2. Percentages of uncleaved α - and β -subunits together with those of their proteolytic fragments (α' and β') after digestion for 30 min at 30 °C with various proteinases

Phosphocellulose-purified tubulin was incubated with 2 % (w/w) *S. aureus* V8 proteinase (V8a); or 1 % (w/w) *S. aureus* V8 proteinase (V8b), or 0.1 % (w/w) proteinase K (pK), or 1.25 % (w/w) subtilisin (S). Also, a sample of undigested tubulin was polymerized in the presence of 10 % (v/v) dimethyl sulphoxide. The percentage of uncleaved subunits and their proteolytic fragments were calculated by densitometry of the photographs obtained from gel electrophoresis of the digested samples by using a P-1700 Optonics microdensitometer.

	Proportion of tubulin subunit or fragment (%)				
	V8a	V8b	pK	S	Pc
α	100	100	79	30	100
α'	—	—	21	70	—
β	36	66	42	25	100
β'	64	34	58	75	—

subtilisin. Digestion of purified tubulin with *S. aureus* V8 proteinase resulted in the appearance of a cleaved polypeptide (β') with an electrophoretic mobility similar to that of subtilisin-digested tubulin β -subunit as well as other proteolytic fragments with a higher electrophoretic mobility than that of the β' -polypeptide (Fig. 1). Digestion of tubulin with proteinase K resulted in the cleavage of both tubulin subunits (α and β), yielding cleaved polypeptides (α' and β') with electrophoretic mobilities similar to those of subtilisin-cleaved tubulin subunits. Proteinase K digestion also produces other proteolytic fragments with a higher electrophoretic mobility than the β' polypeptide (Fig. 1).

A formic acid cleavage analysis of the undigested (α and β) and the digested (α' and β') tubulin subunits was performed as previously indicated (Sonderregger *et al.*, 1982; Serrano *et al.*, 1984a) in order to determine whether the main cleavage site produced by *S. aureus* V8 proteinase or proteinase K digestion of these subunits was near the C-terminal or the N-terminal end (results not shown). The result obtained indicated that *S. aureus* V8 proteinase cleaves the β -subunit at a site identical with or very proximal to that produced by subtilisin cleavage (position 408–409) (Maccioni *et al.*, 1986), whereas the α -subunit was not cleaved at this position. Proteinase K cleaved α - and β -subunits at positions similar to those cleaved by subtilisin (positions 417–418 and 408–409) (Maccioni *et al.*, 1986). Under the conditions used for limited proteolysis, the percentage of α - and β -subunits cleaved at their C-terminal region varied for each proteinase (Table 2). Subtilisin cleaved both α - and β -subunits to a similar extent, whereas proteinase K preferentially, but not exclusively, cleaved the β -subunit, and *S. aureus* V8 proteinase cleaved only the β -subunit. In the case of subtilisin, almost 95 % digestion of α - and β -subunits at their C-terminal ends could be obtained. However, in the case of *S. aureus* V8 proteinase or proteinase K it was not possible to obtain a similar extent of digestion of tubulin subunits under these conditions since α' - and β' -polypeptides were

Fig. 2. Subtilisin-*S. aureus* V8 proteinase double digestion of tubulin

Tubulin (2 mg/ml) digested with subtilisin (S) or with *S. aureus* V8 proteinase as indicated in Fig. 1 legend was polymerized as indicated in the Materials and methods section. The polymerized *S. aureus*-V8-digested protein (V8) at a concentration of 2 mg/ml was digested with subtilisin (1 %, w/w) for 30 min at 30 °C (V8-S), and the polymerized subtilisin-digested tubulin (S) with *S. aureus* V8 proteinase (2 %, w/w) for 30 min at 30 °C (S-V8). The digested proteins were subjected to electrophoresis in a 5–15 % polyacrylamide slab gel. The positions of undigested tubulin subunits (α and β) and those of digested tubulin subunits (α' and β') are indicated.

further degraded to lower-molecular-mass fragments (results not shown). This formic acid mapping was also confirmed by performing double digestions of tubulin. Tubulin was first digested with *S. aureus* V8 proteinase or subtilisin, the digested protein was polymerized and the polymerized protein was further digested with subtilisin in the first case and *S. aureus* V8 proteinase in the second case (Fig. 2). The result of this experiment confirmed the formic acid mapping and indicates that upon *S. aureus* V8 proteinase digestion a conformational change was not produced, since the subtilisin digestion pattern of the *S. aureus*-V8-digested protein is similar to that found with subtilisin digestion of undigested tubulin (Fig. 2).

Effect of limited proteolysis of tubulin on its self-assembly

As indicated above, limited proteolysis of tubulin can affect its polymerization capacity, depending on the proteinase used. Digestion with some proteinases yielded cleaved subunits with a lower polymerization capacity, whereas others increased this capacity. Fig. 3 shows that *S. aureus* V8 proteinase or proteinase K digestion resulted in cleaved subunits with a higher polymerization capacity than that of undigested tubulin, but lower than that of subtilisin-cleaved subunits. *S. aureus*-V8-proteinase- and proteinase-K-cleaved tubulin subunits retained not only their polymerization but also their depolymerization capacity, comparable with subtilisin-digested tubulin. Thus cleaved tubulin may be purified by successive polymerization–depolymerization cycles. Fig. 4 shows

Fig. 3. Kinetics of proteolytic digestion of tubulin

Tubulin (2 mg/ml) (●) was digested with trypsin (■), chymotrypsin (▲), Pronase (◇), subtilisin (□), proteinase K (△) and *S. aureus* V8 proteinase (○) for different times (0–30 min) as indicated in the Materials and methods section. After digestion, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-GTP and 10% (v/v) dimethyl sulphoxide were added to the samples, which were incubated for 15 min at 37 °C. The samples were then centrifuged in a Beckman Airfuge for 5 min at 25 °C, and the amounts of polymerized and unpolymerized protein were quantified by the method of Lowry *et al.* (1951). This Figure shows the percentage of polymerized protein, taking as 100% the amount of undigested tubulin polymer in relationship to the time of digestion.

the electrophoretic pattern of cycled cleaved tubulin, after a previous digestion with subtilisin, proteinase K or *S. aureus* V8 proteinase. Comparison of electrophoretic patterns from Figs. 1 and 4 indicates the selective loss after polymerization–depolymerization cycles of those fragments found in Fig. 1 with a higher electrophoretic mobility than that of the β' -polypeptide.

It has been indicated that upon subtilisin removal of the C-terminal domain of both subunits, the digested tubulin subunits polymerize at a similar critical concentration to that of tubulin plus microtubule-associated proteins (Serrano *et al.*, 1984a; Sackett *et al.*, 1985), and lower than that of undigested tubulin. This result suggests a higher nucleation capacity for subtilisin-cleaved tubulin than that of undigested tubulin (Serrano *et al.*, 1984a). *S. aureus*-V8-proteinase- and proteinase-K-digested tubulin purified by two polymerization cycles exhibited a similar critical concentration, which was higher than that of subtilisin-digested tubulin (1.7 mg/ml compared with 0.4 mg/ml), but lower than that of undigested tubulin (1.7 mg/ml compared with > 5 mg/ml) (Fig. 5).

Fig. 4. Assembly of proteolytically digested tubulin

Subtilisin- (lane a), proteinase-K- (lane b) and *S. aureus*-V8-proteinase- (lane c) digested tubulin were polymerized as indicated in the Materials and methods section. The polymerized protein was fractionated by centrifugation (see the Materials and methods section), resuspended in cold buffer A (4 °C) and centrifuged at 4 °C for 5 min to remove cold insoluble aggregates. The supernatant of the cold centrifugation was subjected to electrophoresis in a 5–15% polyacrylamide slab gel. The positions of uncleaved tubulin subunits (α and β) and of cleaved tubulin subunits (α' and β') are indicated.

Effect of limited proteolysis of tubulin on the shape of assembled tubulin polymers

Two types of proteinases were used, those that decrease (trypsin, chymotrypsin and Pronase) and those that increase (subtilisin, proteinase K and *S. aureus* V8 proteinase) tubulin self-assembly. In the first case dimethyl sulphoxide was added to facilitate polymerization (Himes *et al.*, 1977), but in the second case dimethyl sulphoxide was omitted for polymerization. As shown in Fig. 6, in the presence of dimethyl sulphoxide undigested tubulin-, trypsin- or Pronase-digested tubulin polymerized into microtubules and open structure (the morphology of the latter is probably induced by the addition of dimethyl sulphoxide) (Himes *et al.*, 1977). However, after chymotrypsin digestion, a dramatic change was observed in the morphology of the tubulin polymers, which were composed almost exclusively of extended sheets (see also Fig. 7c). Chymotrypsin produced two cleavage sites on the β -subunit (Fig. 7a, lane b), yielding one fragment of 30 kDa (containing the N-terminus) and two fragments of 21 and 20 kDa (Mandolkow *et al.*, 1985; Serrano & Avila, 1985). Both the 21 kDa and 20 kDa fragments were located at the C-

terminal end of the molecule, as determined by formic acid digestion (Fig. 7b, lane b). The 2 kDa fragment differed from the 20 kDa peptide in only one region of approx 1 kDa, located at the C-terminal extreme of the β -subunit (see below, experiments of subtilisin–chymotrypsin double digestion; Fig. 7a, lane c).

Fig. 5. Critical concentration for the assembly of proteolytically digested tubulin

Tubulin depleted of microtubule-associated proteins (●) was digested with subtilisin (□), proteinase K (△) and *S. aureus* V8 proteinase (○) and cycled twice. Tubulin samples (0.1 ml) were polymerized at different protein concentrations (0.5–5.8 mg/ml) under the conditions indicated in the Materials and methods section. Polymerized protein was quantified and the amount of protein was plotted versus protein concentration present in the incubation mixture before assembly.

To determine whether the dramatic change in morphology of the tubulin polymers upon chymotrypsin digestion was due to the cleavage at position 281–282 of the molecule (which resulted in the appearance of the 30 kDa fragment and 21 kDa C-terminal fragment) or at the C-terminal end (which yielded the 21 kDa fragment and the 20 kDa fragment by cleaving the 1 kDa end region) (Mandelkow *et al.*, 1985; Serrano & Avila, 1985), a double digestion with subtilisin followed by chymotrypsin was performed. After this digestion, the internal cleavage site, but not that at the C-terminal end, was maintained. Assembly of double-digested tubulin resulted in the appearance of structures with a morphology similar to those of subtilisin-digested tubulin polymers and different from those of chymotrypsin-digested tubulin structures (Fig. 7g). This suggests that the morphology of tubulin polymers after chymotrypsin digestion is not determined by internal cleavage of the molecule. A result similar to, but not identical with, that of Fig. 7(g) was found when tubulin was first digested with chymotrypsin and then with subtilisin (Fig. 7f), although in this case an increased number of open structures was found. A further comparison between the morphology of chymotrypsin-digested tubulin (Fig. 7c) and subtilisin–chymotrypsin-double-digested tubulin polymers was then performed by optical diffraction of the electron micrographs obtained from both preparations [see Fig. 7(e) and Serrano *et al.* (1984a)]. As shown in Figs. 7(d) and 7(e), differences were found between both types of polymers, in that the double-digested (subtilisin–chymotrypsin) tubulin polymers (Fig. 7c) exhibited equatorial and longitudinal spacings similar to those described for microtubules (Amos, 1979), but chymotrypsin-digested tubulin polymers did not (Fig. 7d). It is possible that the chymotrypsin-digested tubulin polymers are related to Zn^{2+} -induced tubulin sheets (Crepeau *et al.*, 1978).

Differences were also found in the morphology of the polymers of tubulin assembled after digestion of the protein with proteinases that promote assembly (subtilisin, proteinase K and *S. aureus* V8 proteinase). As shown in Fig. 8, subtilisin-digested tubulin polymers were com-

Fig. 6. Morphology of the assembled polymers of proteolytically (chymotrypsin, trypsin and Pronase) digested tubulin

Undigested tubulin (Pc) (2 mg/ml) or chymotrypsin- (C), trypsin- (T) and Pronase- (P) digested tubulin (2 mg/ml) were polymerized in the presence of 10% (v/v) dimethyl sulphoxide as indicated in the Materials and methods section, and thin sections roughly perpendicular to the polymers were obtained and examined by electron microscopy as indicated by Serrano *et al.* (1984a).

Fig. 7. Subtilisin-chymotrypsin double digestion of tubulin

(a) Twice-cycled subtilisin-digested tubulin (2 mg/ml) was further digested with chymotrypsin as indicated in the Materials and methods section. The double-digested (subtilisin-chymotrypsin) tubulin (lane a) and chymotrypsin-digested tubulin (lane b) were subjected to electrophoresis in a 10% polyacrylamide slab gel. The arrows indicate the positions of the chymotrypsin-digested β -tubulin fragments (30, 21 and 20 kDa) and the double-digested fragment (16 kDa), which lacks the 4 kDa region located at the C-terminal end. (b) The 21 kDa and 20 kDa fragments of (a) were iodinated as previously indicated (Elder *et al.*, 1977), digested with formic acid (see the Materials and methods section) and subjected to electrophoresis in a 10–20% polyacrylamide slab gel. The arrows indicate the position of the uncleaved fragments (21 and 20 kDa) and the formic acid-cleaved fragments of both peptides (β_3 and β_2) (Serrano *et al.*, 1984a). (c) Tubulin (2 mg/ml) was digested with chymotrypsin for 8 min at 30 °C and polymerized in the presence of 10% (v/v) dimethyl sulphoxide as indicated in the Materials and methods section. Samples of the incubation mixture were placed on carbon/collodion-coated copper grids and stained with 1% (w/v) uranyl acetate. The bar represents 100 nm. (d) Optical diffraction of a region of the chymotrypsin-digested tubulin polymer shown in (c). The bar represents 5 nm⁻¹. (e) Optical diffraction of a subtilisin-chymotrypsin-double-digested tubulin polymer (not shown). The bar represents 5 nm⁻¹. (f) Tubulin (2 mg/ml) was digested with chymotrypsin as indicated in (c), cycled once, and then digested with subtilisin before polymerization in the presence of 10% (v/v) dimethyl sulphoxide. (g) Tubulin processed as in (f) except that the order of the proteinase digestions was reversed.

Fig. 8. Morphology of the assembled polymers of proteolytically (subtilisin, proteinase K and *S. aureus* V8 proteinase) digested tubulin

Microtubule protein (MT) (2 mg/ml) or twice-cycled subtilisin- (S), proteinase-K- (PK) and *S. aureus*-V8-proteinase- (V8) digested tubulin (2 mg/ml) were polymerized in the absence of dimethyl sulphoxide and processed as indicated in Fig. 6 legend.

Table 3. Distribution of polymer structures found after limited proteolysis of tubulin

Phosphocellulose-purified tubulin (Pc) was incubated with 2% (w/w) *S. aureus* V8 proteinase (V8a), or 1% (w/w) *S. aureus* V8 proteinase (V8b), or 0.1% (w/w) proteinase K (PK), or 1% (w/w) subtilisin (S). The samples were then polymerized in the presence of 10% (v/v) dimethyl sulphoxide (undigested tubulin) or in its absence (digested tubulin). Thin sections, roughly perpendicular to the polymers, were examined by electron microscopy and photographs were taken. Around 450 different polymers from each sample were classified by their morphology and the percentage of each class is indicated. The polymers in which a great number of hooks were present, making any classification impossible, are indicated by MHF. The asterisk (*) indicates those polymers in which the hooks were oriented in opposite direction.

Morphology	Distribution of polymer structures (%)				
	V8a	V8b	PK	S	Pc
o	48.8	91.7	10	1.3	82.4
o	20.7	—	4.4	—	9
o	9.1	5.8	2.9	15.2	4.9
o*	12	—	—	—	3.2
c	1.8	—	2.2	0.6	—
o	0.2	—	14.5	7.6	—
o	0.6	1	0.7	2.1	—
o*	2.9	1	—	—	—
o*	0.6	0.5	14.5	—	—
MHF _A	—	—	—	73	0.4
MHF _B	3.1	—	76.6	—	—

posed mainly of multiple hooked microtubules, all the hooks having 11 protofilaments and the same directional growth (Heidemann & McIntosh, 1980), whereas *S. aureus* V8 proteinase-digested tubulin polymers were composed mainly of closed polymers and few simple hooked polymers in which the hooks could grow bidirectionally. Proteinase-K-digested tubulin polymers were composed primarily of multiple hooked polymers,

which were different from those assembled from subtilisin-digested tubulin (Fig. 8), since the hooks grew in any direction, with no apparent organization.

Table 3 shows the distribution of the different polymer structures found after limited proteolysis of tubulin with the three proteinases (subtilisin, *S. aureus* V8 proteinase and proteinase K).

DISCUSSION

Limited proteolysis of tubulin with different proteinases has indicated the existence of defined regions in both α - and β -subunits of tubulin that are susceptible to proteolysis. In the α -subunit there are three regions, one located in the first third of the molecule (around position 133–134) (Maccioni & Seeds, 1983; Serrano *et al.*, 1986), another in the second third (around positions 320–321 and 339–340) (Serrano *et al.*, 1984b; Mandelkow *et al.*, 1985), and the last near the C-terminal end (position 417–418) (Serrano *et al.*, 1984a,c; Sackett *et al.*, 1985; Maccioni *et al.*, 1986). In the β -subunit these regions are in the first third of the molecule (around position 131–132) (Serrano *et al.*, 1986), in the second third (position 281–282) (Mandelkow *et al.*, 1985; Serrano & Avila, 1985), near the C-terminal end (position 408–409) (Serrano *et al.*, 1984a,b; Sackett *et al.*, 1985; Maccioni *et al.*, 1986) and, as demonstrated above and as previously indicated by Mandelkow *et al.* (1985), at about ten residues from the C-terminus of the β -subunit (Fig. 9).

Cleavage at the first or second thirds of α - and β -subunits resulted in a decrease in the tubulin polymerization capacity when Mes was used as buffer (Brown & Erickson, 1983; Maccioni & Seeds, 1983; Serrano *et al.*, 1984b).

Removal of the 40 residues located at the C-terminal region of the β -subunit (*S. aureus* V8 proteinase) or of both tubulin subunits by subtilisin resulted in an increase of the initiation of tubulin polymerization (nucleation) and in an increase of lateral interactions between tubulin protofilaments. Erickson & Pantaloni (1980) proposed a simple model for nucleation of tubulin polymers, which involved longitudinal and lateral interactions; the latter

Fig. 9. Peptide map obtained by formic acid mapping of the digested tubulin subunits

The main cleavage sites (|) of trypsin (■), chymotrypsin (▲), Pronase (◆), subtilisin (□), proteinase K (△) and *S. aureus* V8 proteinase (○) are indicated. The different rectangular zones indicate the different effects produced on tubulin polymerization or on the polymer morphology, if these regions are cleaved by a proteinase: | inhibits tubulin polymerization; ■ facilitates the assembly of tubulin, promotes lateral interaction between polymers and affects the directional growth of hooks on a microtubule; □ changes the arrangement of tubulin subunits on the polymer. Deletion of ■ from the β -subunit results in a decrease of the critical concentration and in the appearance of a few hooked polymers that could grow in opposite directions. The inset shows details of the different effects on the polymer morphology by digestion with subtilisin (S), *S. aureus* V8 proteinase (V8) and chymotrypsin (C), and a detail of undigested tubulin polymers (Tb).

interactions are the limiting step for polymer initiation. The increase in lateral interactions after removal of C-terminal region was observed by the formation of hooks. These hooks resembled those polymerized in the presence of dimethyl sulphoxide, detergent and salt and that could define microtubule directionality (Heidemann & McIntosh, 1980).

The results obtained on the morphology of the polymer after limited proteolysis of tubulin with subtilisin, *S. aureus* V8 proteinase or proteinase K indicated that in order to obtain the dramatic increase in lateral interactions between polymers it is necessary to remove the C-terminal region of both α - and β -subunits (Table 3). The removal of only those residues located at the C-terminal end of β -subunits could result in the appearance of bidirectional hooked structures. Finally, the most different polymers observed with respect to microtubules were those found after limited proteolysis of tubulin with chymotrypsin. As we have indicated by double-digestion experiments with subtilisin and chymotrypsin, removal of about ten amino acid residues of the C-terminal region of the β -subunit resulted in the appearance of open sheets with a variable number of protofilaments, in which the arrangement of tubulin subunits was different from that found in microtubules, and probably resembled that of Zn^{2+} -induced tubulin sheets. This result contradicts that described for chymotryptic digestion of tubulin by Mandelkow *et al.* (1985), in which they found normal dimethyl sulphoxide-induced tubulin polymers

after chymotryptic digestion of tubulin (Himes *et al.*, 1977).

Mandelkow *et al.* (1985) used a lower enzyme/tubulin ratio and a lower temperature for chymotrypsin digestion than those used in this work, conditions that could have resulted in a lower proportion of cleaved tubulin and, consequently, in the formation of polymers similar to those found for dimethyl sulphoxide-induced polymers from undigested tubulin (Himes *et al.*, 1977). Moreover, they purified tubulin by a different method and stored it in sucrose at -70°C . Recently it has been reported that the shape of the tubulin dimer in the microtubule is changed upon adding sucrose to the polymerizing buffer (Beese *et al.*, 1987). Consequently the incubation of tubulin with sucrose before digestion could result in a conformational change and in the appearance of polymers with different morphology.

These results also suggest that the C-terminal region of tubulin could be divided into at least two regions, one that comprises the last ten amino acid residues of the β -subunit and seems to be involved in the arrangement of the subunits and in the curvature of the polymer, and a second that comprises the last 40 amino acid residues of both subunits and seems to be involved in the closure, directional growth, nucleation of microtubules and lateral interactions between polymers (Fig. 9). These results are also of interest in the light of the fact that the most variable region between isotubulins of the same species in vertebrates is the region comprising the last 15

residues of the α - and β -subunits (Sullivan & Cleveland, 1983; Villasante *et al.*, 1986); this region is conserved in the same isotubulin peptides among different vertebrate species (Sullivan & Cleveland, 1983; Villasante *et al.*, 1986). These data imply that the C-terminal region of tubulin is a regulatory domain involved in the polymerization of tubulin and in the morphology of tubulin polymers. It contains completely conserved regions at residues 390–437 of the α -subunit and residues 380–427 of the β -subunit, which are probably involved in fundamental aspects of microtubule functions, and variable regions (from the final amino acid residues of the conserved regions to the C-termini of the tubulin subunits) that probably confer functional specificity to the isotubulins.

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REFERENCES

- Amos, L. A. (1979) in *Microtubules* (Roberts, K. & Hyams, J. J., eds.), pp. 2–64, Academic Press, New York
- Beese, L., Stubbs, G., Thomas, J. & Cohen, C. (1987) *J. Mol. Biol.* **196**, 575–580
- Brown, R. H. & Erickson, H. (1983) *Arch. Biochem. Biophys.* **220**, 46–51
- Crepeau, R. H., McEwan, E. & Edelstein, S. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5006–5010
- Crestfeld, D. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
- Elder, J. R., Hampton, J. & Lerner, R. (1977) *J. Biol. Chem.* **252**, 6510–6515
- Erickson, H. P. & Pantaloni, D. (1980) in *Microtubules and Microtubule Inhibitors* (De Brabander, M. & De Meyer, J., eds.), pp. 119–132, Elsevier, Amsterdam
- Fairbanks, G., Steck, N. C. & Wallach, D. F. (1971) *Biochemistry* **10**, 2606–2617
- Heidemann, S. R. & McIntosh, J. R. (1980) *Nature (London)* **286**, 517–519
- Himes, H. R., Burton, R. P. & Gaito, J. M. (1977) *J. Biol. Chem.* **252**, 6222–6226
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Love, M., Millary, D. & Huston, S. (1981) *Arch. Biochem. Biophys.* **207**, 300–310
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maccioni, R. B. & Seeds, N. W. (1983) *Biochemistry* **22**, 1567–1572
- Maccioni, R. B., Serrano, L., Avila, J. & Cann, R. (1986) *Eur. J. Biochem.* **156**, 375–381
- Mandelkow, E. M., Hermann, M. & Rühl, U. (1985) *J. Mol. Biol.* **85**, 311–313
- Sackett, D. L., Battachatyya, B. & Wolff, J. (1985) *J. Biol. Chem.* **260**, 43–45
- Serrano, L. & Avila, J. (1985) *Biochem. J.* **230**, 551–556
- Serrano, L., de la Torre, J., Maccioni, R. B. & Avila, J. (1984a) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5989–5993
- Serrano, L., Avila, J. & Maccioni, R. B. (1984b) *J. Biol. Chem.* **259**, 6607–6611
- Serrano, L., Avila, J. & Maccioni, R. B. (1984c) *Biochemistry* **23**, 4675–4681
- Serrano, L., Wandosell, F. & Avila, J. (1986) *Anal. Biochem.* **159**, 253–259
- Shelanski, M. L., Gaskin, F. & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 765–768
- Sonderregger, P., Jansi, R., Genning, H., Brunschweiler, K. & Christien, P. (1982) *Anal. Biochem.* **122**, 298–301
- Sullivan, K. F. & Cleveland, D. W. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4327–4331
- Villasante, A., Wang, D., Dobner, P., Dolph, P., Lewis, S. A. & Cowan, M. J. (1986) *Mol. Cell. Biol.* **6**, 2409–2419
- Weingarten, M. D., Lockwood, H. H., Hwo, S. Y. & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1858–1862

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