

# Tubulin, Hybrid Dimers, and Tubulin S

## STEPWISE CHARGE REDUCTION AND POLYMERIZATION\*

(Received for publication, November 14, 1984)

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Limited proteolysis of rat brain tubulin ( $\alpha\beta$ ) by subtilisin cleaves a 1–2-kDa fragment from the carboxyl-terminal ends of both the  $\alpha$  and  $\beta$  subunits with a corresponding loss in negative charge of the proteins. The  $\beta$  subunit is split much more rapidly (and exclusively at 5 °C), yielding a protein with cleaved  $\beta$  and intact  $\alpha$  subunit, called  $\alpha\beta_S$ , which is of intermediate charge. Further proteolysis cleaves the carboxyl terminus of the  $\alpha$  subunit leading, irreversibly, to the doubly cleaved product, named tubulin S, with a composition  $\alpha_S\beta_S$ . Both cleavage products are polymerization-competent and their polymers are resistant to 1 mM  $\text{Ca}^{2+}$ - and 0.24 M NaCl-induced depolymerization. The two polymers differ in that the  $\alpha\beta_S$  polymer is stable to cold, GDP, and podophyllotoxin, whereas tubulin S polymer is disassembled by these agents; moreover,  $\alpha\beta_S$  forms ring-shaped polymers, whereas  $\alpha_S\beta_S$  forms filaments associated into bundles and sheets. Tubulin S co-polymerizes with native tubulin yielding a mixed product of intermediate stability. The presence of low mole fractions of tubulin S leads to a marked reduction in the critical concentration for polymerization of the mixture.

Covalent modifications of tubulin at SH groups (1–6), basic amino acids (7–9), and tryptophan (10), as well as by iodination (11, 12), all have resulted in loss of polymerizability but have provided few clues regarding the contribution of the altered groups to polymerization. On the other hand, limited proteolysis has provided some surprising insights into the contributions of different portions of the  $\alpha$  and  $\beta$  subunits. Limited proteolysis by thermolysin or chymotrypsin yields  $\alpha$  and  $\beta$  subunits cleaved at a single site, respectively. Trypsin cleaves the  $\alpha$  subunit at two sites. The proteolyzed tubulin is competent to polymerize if buffers are appropriately chosen (13). Chymotryptic fragments form somewhat abnormal looking microtubules but retain the ability to bind colchicine (14). By contrast, cleavage of both subunits at a site near the C terminus with subtilisin actually stimulates polymerization of the resulting protein and lowers the critical concentration for assembly by more than an order of magnitude (15). The cleaved protein is much reduced in negative charge and it was suggested that charge-charge repulsion between adjacent tubulin molecules inhibits polymerization. This repulsion is relieved by removal of the C-terminal fragments from the  $\alpha$  and  $\beta$  subunits. The C-terminal end of each subunit is thus

seen as the locus of action of polymerization-modulating agents such as microtubule-associated proteins or polycations. In this paper we characterize the tubulin S<sup>1</sup> polymer formed by subtilisin treatment and the co-polymerization of cleaved with intact tubulin; we also describe the properties of an intermediate form of tubulin, possessing one intact and one cleaved C terminus.

### MATERIALS AND METHODS

**Microtubule Protein**—The protein was prepared from brains of ~120-g male Sprague-Dawley rats. Brains were rinsed and then homogenized in 1:1 (v/w) Mes<sup>2</sup> assembly buffer (0.1 M Mes, 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM GTP, pH 6.7). Microtubule protein was purified by three cycles of temperature-dependent assembly and disassembly (17). Following this, the protein was either further purified as described below or drop frozen in liquid nitrogen and stored at –70 °C.

**Purification of Tubulin**—Tubulin was purified from microtubule protein by ion exchange chromatography on phosphocellulose. Approximately 80 mg of microtubule protein was applied to a column (1.5 × 13.5 cm) of phosphocellulose previously saturated with  $\text{MgSO}_4$  and equilibrated with column buffer (50 mM Pipes, 1 mM  $\text{MgSO}_4$ , 2 mM EGTA, pH 6.9). The column was developed with the same buffer at a flow rate of ~0.3 ml/min (18). Tubulin eluted from this column was >98% pure as judged by densitometry of Coomassie Blue-stained SDS gels. Tubulin was concentrated to >5 mg/ml using Amicon CF50A membrane cones, drop frozen in liquid nitrogen, and stored at –70 °C.

**Digestion of Tubulin**—This was performed with subtilisin BPN (19) at a concentration of 1% (w/w) tubulin. Enzyme stock solution was prepared by dissolving subtilisin at 1 mg/ml in water. This was frozen in aliquots which were stored at –70 °C and thawed once only. Digestion was performed in assembly buffer containing 1 mM GTP at the temperatures indicated in the text. The reaction was terminated by the addition of 1% by volume of 1% (w/v) PMSF in dimethyl sulfoxide.

**Polymerization**—This was monitored by turbidity at 350 nm using a Cary Model 219 spectrophotometer. The sample chamber was thermostatically controlled to  $\pm 0.1$  °C using a Lauda K2-R circulator. Polymerization was at 37 °C except as noted in the text. The optical density was a linear function of the mass of polymer as determined by pelleting either directly or after treatment with glutaraldehyde according to Ref. 20. Each polymer exhibited a different optical density yield per mg of pelleted protein; hence the optical density values are not strictly comparable (21). Nevertheless, each polymer scattered light in a concentration-dependent manner and the progress curves can be interpreted as the appearance and disappearance of the different polymers.

**Electrophoresis**—Electrophoresis of protein samples was performed

<sup>1</sup> In conformity with ribonuclease S (16), we have named the subtilisin-cleaved tubulin as tubulin S even though the S peptide is not retained on the S protein after bond cleavage.

<sup>2</sup> The abbreviations used are: Mes, 2-(N-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

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using native and denaturing gel systems. Native proteins were separated by agarose electrophoresis in 2-mm-thick 1% agarose gel slabs (electrophoresis grade agarose, Bethesda Research Laboratories) prepared in assembly buffer and cast on Gelbond (FMC). Experimental samples were prepared for electrophoresis by mixing 1:1 with 20% glycerol in assembly buffer containing 0.002% bromphenol blue. At this point, samples were kept on ice and either run the same day or quickly frozen in microcentrifuge tubes using dry ice, stored at  $-70^{\circ}\text{C}$ , and run up to several weeks later with no change in pattern. Gels were run at  $5\text{--}10^{\circ}\text{C}$  submerged in assembly buffer, which was recirculated during electrophoresis. Separation time was  $\sim 2$  h at 60 V for a gel of  $10 \times 7$  cm using a Mini-sub cell (Bio-Rad). Gels were stained with Coomassie Brilliant Blue R-250, destained with 25% methanol, 10% acetic acid, washed in water, and air-dried.

Subunit composition was analyzed by sodium dodecyl sulfate electrophoresis. Separation was performed in slab gels, using a modification of the method of Laemmli (22). 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 Sigma lauryl sulfate or the equivalent (23). Following electrophoresis, the gels were stained and destained as the agarose gels and air-dried after soaking in 3% glycerol in water.

The time course of digestion of tubulin was quantitated by densitometry of stained gels. This was performed on dried gels using an LKB 2202 Laser Densitometer equipped with a Model 2220 Recording Integrator. The intensity of each band was normalized to the total absorbance in each scan in order to eliminate variations due to loading volume.

**Electron Microscopy**—This was performed on aliquots taken from polymerization reactions which were being monitored turbidimetrically. Samples were mixed 1:1 with prewarmed 4% glutaraldehyde in assembly buffer,  $\sim 10\ \mu\text{l}$  applied to a carbon/Formvar-coated grid for  $\sim 30$  s. Grids were then stained with several drops of 1% uranyl acetate. Excess liquid was drawn off with a filter paper and the grids were air-dried. Samples were examined with a Zeiss Model 10A electron microscope.

Protein concentration was determined by the method of Lowry *et al.* (24). GTP, Mes, Pipes, EGTA, PMSF, subtilisin-BPN, and glycerol were from Sigma. Dimethyl sulfoxide was spectrophotometric grade from Aldrich. All other chemicals used in this study were reagent grade.

## RESULTS

When the time course of subtilisin cleavage of tubulin is followed under conditions that permit polymerization of the products to occur, two optical density peaks are observed (Fig. 1). There is a rapid initial increase in turbidity after enzyme addition followed by a decrease and, after some time, a second increase that is invariably greater than the first peak. The first peak is not a constant fraction of the second and is not the result of simple protein-protein interaction between enzyme and tubulin, since addition of PMSF-inactivated subtilisin fails to bring about these turbidity changes. It should also be noted that the valley between the two optical density peaks never attains the original baseline value.

When subtilisin digestion is monitored by SDS-gel electrophoresis (Fig. 2A), it is observed that the  $\beta$  subunit is cleaved very much faster than the  $\alpha$  subunit. Densitometric scans of these gels allow quantitation of this difference (Fig. 3A). The appearance of the first peak corresponds in time to the conversion of  $\beta$  to the slightly smaller molecule which we have termed  $\beta_s$  which is decreased in size by about 2000 Da (15). At the time corresponding to the maximum absorbance of the first peak ( $\sim 2$  min), approximately 80% of the protein is of the form  $\alpha\beta_s$  (lanes 2 and 3). Formation of the second optical density peak corresponds to the conversion of the  $\alpha$  subunit to a form that is decreased in size by  $\sim 2$  kDa which we have termed  $\alpha_s$ . At the time corresponding to the second peak (30 min),  $\sim 80\%$  of protein is converted to the  $\alpha_s\beta_s$  form (lanes 5 and 6). If incubation is continued for a prolonged period, a secondary subtilisin cleavage site is cut, resulting in the gradual appearance of smaller fragments on SDS gels (Fig.

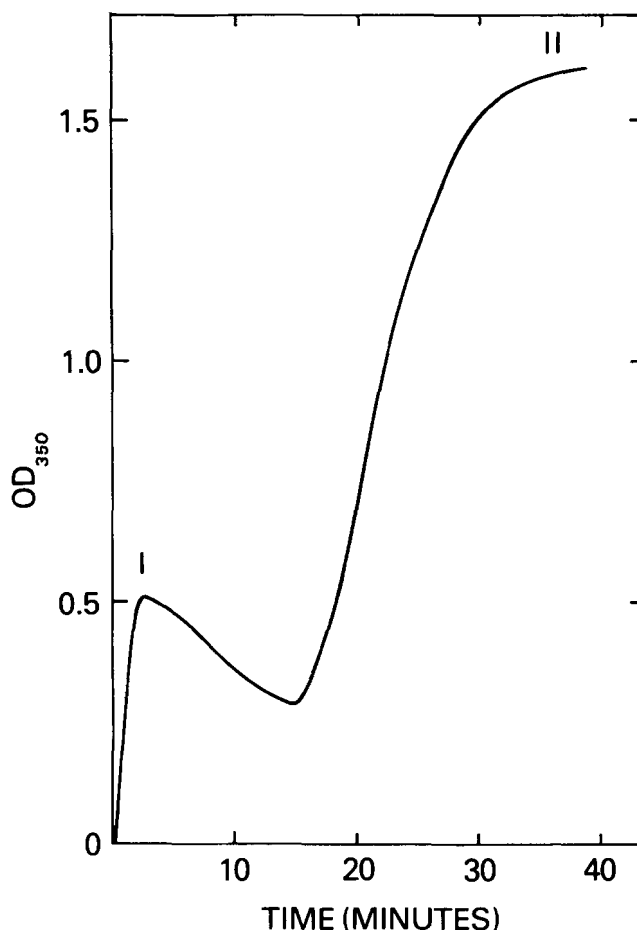


FIG. 1. A typical progress curve of digestion of tubulin by subtilisin. Tubulin (2 mg/ml) in assembly buffer containing 2 mM GTP was incubated with subtilisin (1% w/w) at  $30^{\circ}\text{C}$ .

2A, lanes 7 and 8). These fragments appear to be similar to those recently reported by Serrano *et al.* (25) and are accompanied by a decreased ability to assemble even in the presence of taxol. Note that the temperature for the first three figures was  $30^{\circ}\text{C}$  to facilitate sampling. Subsequent experiments have been carried out at  $37^{\circ}\text{C}$ .

The conversion from  $\alpha\beta$  to  $\alpha_s\beta_s$  can also be monitored by agarose electrophoresis because the  $\alpha\beta$ ,  $\alpha\beta_s$ , and  $\alpha_s\beta_s$  forms differ substantially in charge (Fig. 2B). Again it is seen that the form with intermediate charge corresponds to the first peak of absorbance and the final form corresponds to the second peak. When agarose bands are excised and run on an SDS gel, the intermediate band yields  $\alpha$  and  $\beta_s$  bands and the final form yields  $\alpha_s$  and  $\beta_s$  bands (data not shown). Densitometric scans of agarose gels (Fig. 3B) clearly show three stages of the cleavage reaction. The first is the initial state, in which all of the protein is native or  $\alpha\beta$ . This period is brief and corresponds to the brief lag before polymerization. In the second stage, most of the protein is of intermediate charge, has subunit composition  $\alpha\beta_s$ , and corresponds to the first peak of absorbance in Fig. 1. In the third stage, all of the protein is converted to the low charge form, tubulin S, of subunit composition  $\alpha_s\beta_s$ . This corresponds to the second peak of optical density, the high plateau of Fig. 1.

### Polymer Structure

Fig. 4 presents electron micrographs of polymers from peaks I and II. The peak I material is clearly different in structure from peak II and both are different from microtubules. Panel

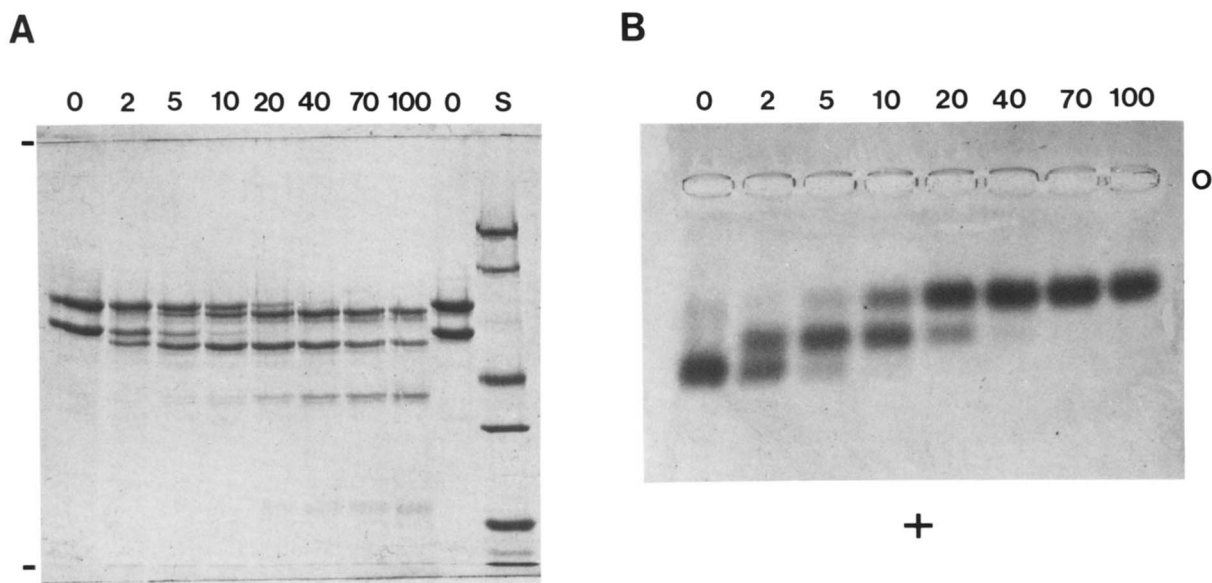
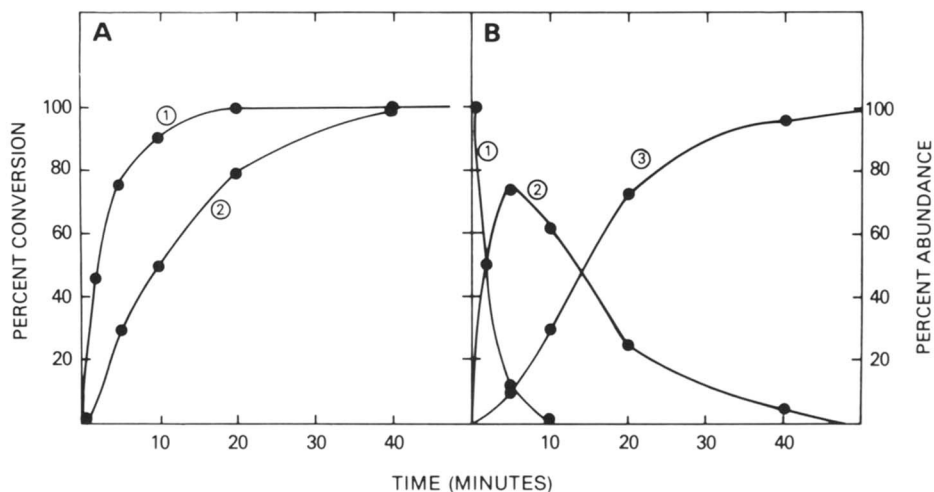


FIG. 2. **The time course of tubulin digestion by subtilisin: electrophoretic analysis.** Tubulin (2 mg/ml) in assembly buffer containing 2 mM GTP was incubated at 30 °C with 1% w/w subtilisin. At the indicated times, aliquots were removed, enzyme was inactivated by addition of PMSF, and all samples were placed on ice. Following completion of digestion, samples were processed for denaturing electrophoresis (SDS gel, panel A) or native electrophoresis (agarose gel, panel B). Numbers at the top refer to minutes of incubation. S = molecular weight standards: phosphorylase (96,000), bovine serum albumin (66,000), ovalbumin (46,000), carbonic anhydrase (29,000), and soybean trypsin inhibitor (21,500).

FIG. 3. **The kinetics of cleavage of the  $\alpha$  and  $\beta$  subunits of tubulin.**

A, the SDS gel in Fig. 2 was scanned and for each time point, the percentage of each subunit present as the intact or cleaved form was calculated. The per cent cleaved is plotted. Curve 1,  $\beta$  subunit; curve 2,  $\alpha$  subunit. B, the agarose gel of Fig. 2 was scanned and for each time point the distribution of protein among the three bands was determined. The per cent of total protein is plotted for: 1, the most rapidly migrating band ( $\alpha\beta$ ); 2, the band with intermediate mobility ( $\alpha\beta_S$ ); and 3, the most slowly migrating band ( $\alpha_S\beta_S$ ).



A shows the  $\alpha\beta_S$  polymer which produces the turbidity of peak I. It is composed of both closed and incomplete rings. The most common closed rings are  $38 \pm 2$  nm in outer diameter ( $27 \pm 2$  nm inner diameter) and appear to be double-walled. Single rings of this size are also observed. A few smaller rings, diameter  $28 \pm 2$  nm (inner diameter  $16 \pm 2$  nm) are also seen. Portions of some double-walled rings of both sizes appear to be single-walled. It is worth emphasizing that these cold-insensitive rings form directly from dimeric tubulin following cleavage of the  $\beta$ -terminus, *i.e.* neither microtubule-associated proteins nor prior formation of microtubules is required.

The polymer in peak II (Fig. 4, panel B), which consists largely of  $\alpha_S\beta_S$  tubulin, is quite different and is composed of filaments which associate into bundles and sheets and, occasionally, tubules. The filaments are  $3.9 \pm 0.1$  nm in diameter (measured center to center on adjacent filaments) and are composed of subunits with a center to center spacing along the filament of  $4.0 \pm 0.1$  nm. Adjacent filaments align such that a line connecting the centers of subunits in adjacent

filaments forms an angle of  $10^\circ$  with the perpendicular to the axis of the filament (see *inset* to Fig. 4). This corresponds to the  $10^\circ$  pitch of the three-start helix of microtubules. Thus, these filaments are quite similar to protofilaments of tubulin in other structures by size, substructure, and interaction of neighboring filaments (26).

Polymers of different morphologies may have rather different light scattering properties (21). Thus, the wavelength dependence of turbidity, as well as the yield of turbidity at a given wavelength, can differ for polymers of spherical, tubular, or sheet-like geometry. Since the turbidity in these experiments is higher than that seen with normal microtubules and since the polymers are obviously different by electron microscopy, we examined the details of turbidity yield for the polymers of peak I and peak II. Peak I polymer was found to scatter light in proportion to  $\lambda^{-2.4}$ , while the value for peak II is  $\lambda^{-2.2}$ . Both values are substantially lower than the  $\lambda^{-3}$  value expected for microtubules, consistent with the electron microscopy data. In addition, the yield of turbidity was examined

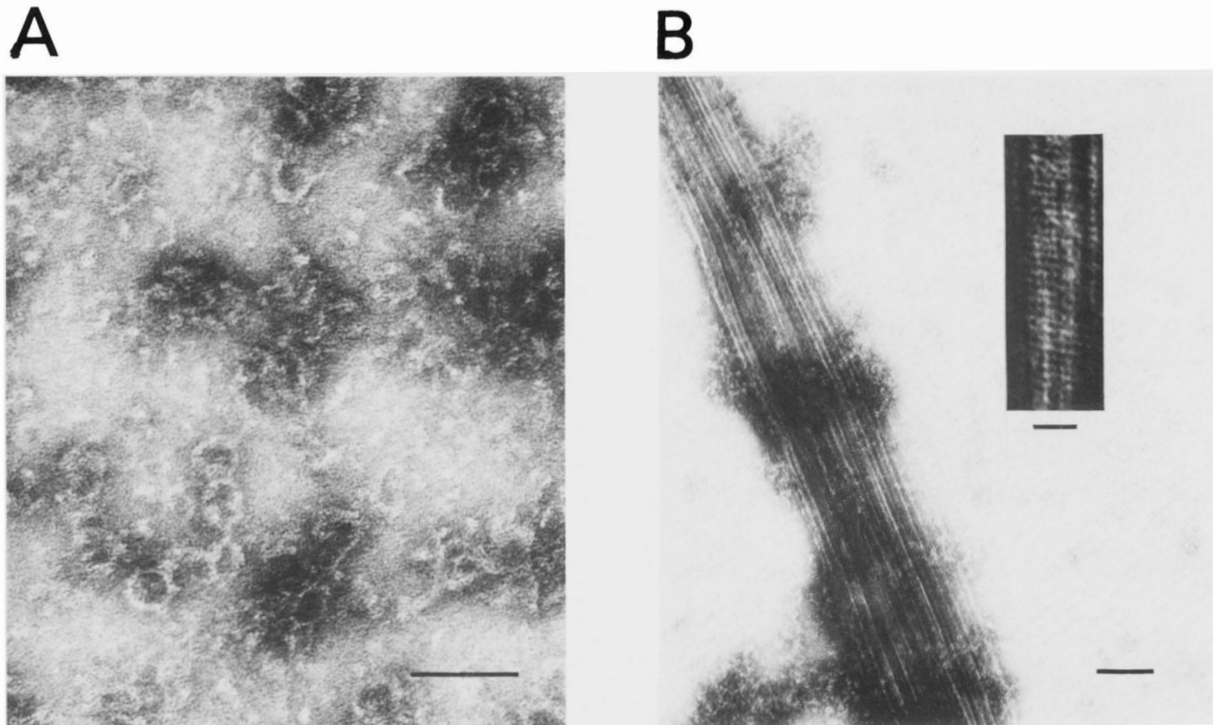


FIG. 4. **Morphology of the polymers.** Tubulin (1 mg/ml) and subtilisin (0.01 mg/ml) were mixed, and the absorbance was followed as in Fig. 1. When the first peak of absorbance was reached, aliquots were removed, fixed, and processed as described under "Materials and Methods" (A). When the second peak of absorbance was reached (representing assembly of fully cleaved tubulin), samples were again removed, fixed, and processed for microscopy (B). Bar = 0.1  $\mu\text{m}$  in panels A and B and 0.02  $\mu\text{m}$  in the inset.

for both polymers and was found to be a linear function of concentration in the range used here. Peak II was found to give a value of 1.8 for  $A_{350}^{1\text{cm}}$ /mg of pellet protein/ml. Peak I gave values of 2.1 while microtubules yielded 0.4.

#### The $\alpha\beta_s$ Intermediate

The difference in rates of cleavage of  $\alpha$  and  $\beta$  tubulin permits a comparison of the polymerization properties of tubulin with only one ( $\alpha\beta_s$ ) or both C termini cleaved ( $\alpha_s\beta_s$ ) simply by stopping the reaction at the appropriate time. Formation of intermediate polymer is dependent on active enzyme, consistent with the suggestion from time course studies that this polymer is due to  $\alpha\beta_s$ . If the enzyme is treated with PMSF prior to the addition of tubulin, no increase above the initial absorbance occurs. Progression from peak I to peak II also requires active enzyme (Fig. 3). Thus, if the enzyme is inactivated by addition of PMSF after the maximum absorbance is achieved for peak I, no further change in absorbance occurs and the  $\alpha\beta_s$  polymer is remarkably stable, as will be detailed below.

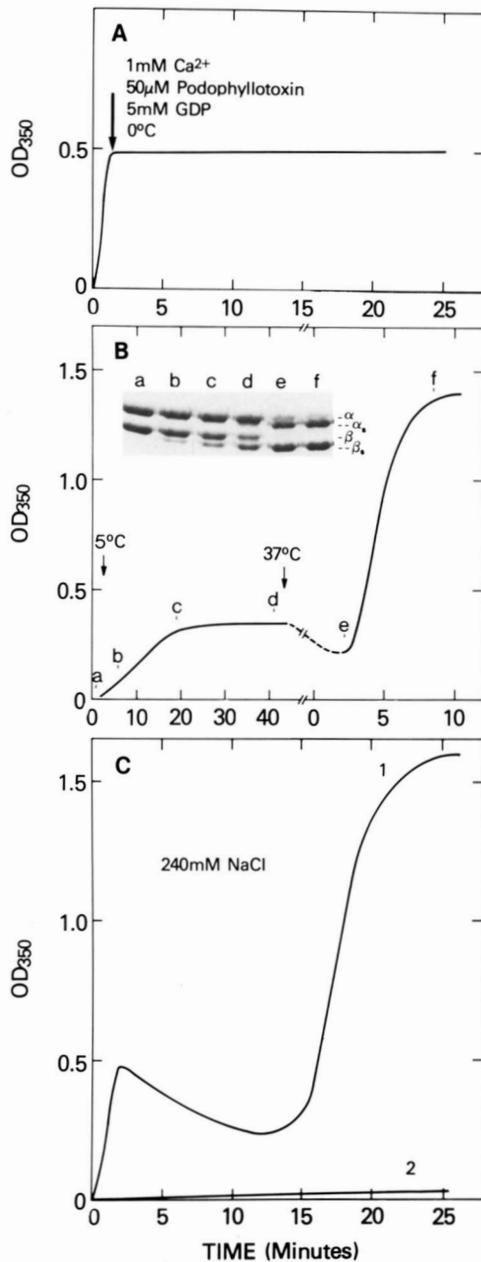
**Cold Sensitivity**—When subtilisin digestion is permitted to proceed until the first peak is attained and the mixture is then rapidly cooled to 0 °C, no depolymerization occurs (Fig. 5A). This is in marked contrast to the effect of cold on the microtubules formed from the starting material. Moreover, digestion of the  $\beta$  subunit by subtilisin proceeds at 5 °C, albeit more slowly, and the  $\alpha\beta_s$  product so formed polymerizes at this temperature, as shown in Fig. 5B. An analysis of the subtilisin digestion of tubulin at 5 °C is also shown in Fig. 5B. Over the time span shown, the  $\beta$  subunit of tubulin is progressively cleaved to  $\beta_s$ , whereas there is no cleavage of the  $\alpha$  subunit detectable at this temperature. If digestion is allowed to continue by rewarming the solution, the progress curve resembles that depicted in Fig. 1. The initial loss of peak I

and the gradual formation of peak II polymer parallels digestion of  $\alpha$  to  $\alpha_s$  (Fig. 5B).

**Sensitivity to Depolymerizing Agents**—Unlike the native rat brain tubulin used as starting material, the  $\alpha\beta_s$  (peak I) polymer is remarkably resistant to depolymerizing agents. It is not depolymerized by the addition of colchicine or podophyllotoxin (50  $\mu\text{M}$ ) or 1 mM  $\text{Ca}^{2+}$  (Fig. 5A). Moreover, the formation of this polymer is not inhibited by high concentrations of salts (0.24 M NaCl), as shown in Fig. 5C. Again this is in marked contrast to intact tubulin.

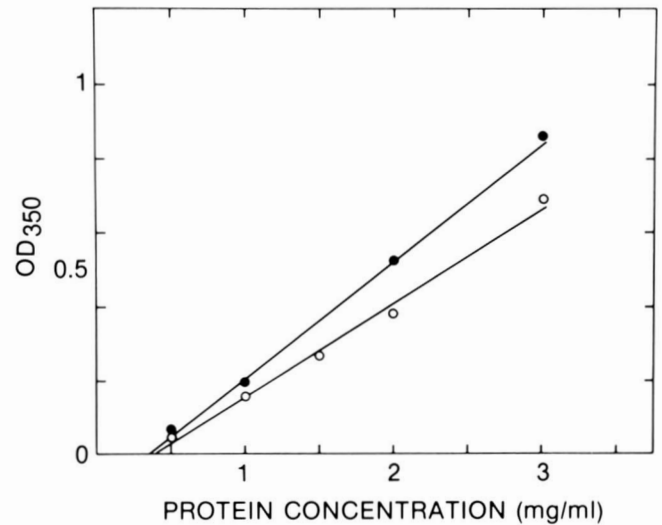
**GTP Requirement**—GTP is required for the assembly of microtubules and certain other polymers of tubulin (21). It was, therefore, important to know whether the polymers formed from subtilisin-treated tubulin showed a similar requirement. Progress curves carried out in the absence of added GTP revealed the formation of peak I polymer but not of peak II polymer in the time span in which this second peak normally forms in the presence of GTP (15). Furthermore, GDP, which is known to promote microtubule depolymerization (21), had no effect on the  $\alpha\beta_s$  polymer when added at 5 mM concentration (Fig. 5A).

**Critical Concentration**—The great stability of the  $\alpha\beta_s$  polymer led us to ask whether or not factors other than further digestion with subtilisin could disassemble the polymer. Reversibility was shown by dilution experiments (27) of preparations cleaved to the maximal optical density of peak I ( $\alpha\beta_s$  polymer—see Fig. 1) and then stabilized against further hydrolysis by treatment with PMSF. As shown in Fig. 6, loss of optical density exceeded that due to dilution alone and yielded an apparent critical concentration for the  $\alpha\beta_s$  polymer of 0.38 mg/ml. This value is virtually identical, within the error of the method, to the critical concentration (0.34 mg/ml) measured in the "forward" direction by addition of increasing concentrations of  $\alpha\beta_s$ . The residual intact tubulin could con-



**FIG. 5. The effect of temperature, salt, and microtubule inhibitors on subtilisin-induced polymerization of tubulin.** *Panel A*, tubulin (1.2 mg/ml) in assembly buffer containing 1 mM GTP was incubated with subtilisin (1% w/w) at 37 °C. 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 µM, or calcium was added to 1 mM. In all cases the reaction was stopped with PMSF at the peak. *Panel B*, tubulin (1.2 mg/ml) in assembly buffer containing 1 mM GTP was incubated with subtilisin (1% w/w) at 5 °C and transferred to 37 °C at the time indicated by the arrow. The inset shows the tubulin region of SDS gels from samples taken at different time intervals during the subtilisin proteolysis and arrested with PMSF. The letters refer to the time points indicated on the progress curve. *Panel C*, tubulin was incubated at 37 °C in assembly buffer containing 1 mM GTP and 240 mM NaCl. In curve 1, the protein concentration was 1.2 mg/ml and polymerization was initiated by addition of subtilisin (1% w/w). In curve 2, polymerization of 3 mg/ml phosphocellulose-tubulin is shown in the presence of identical salt concentrations. This protein concentration allows polymerization in the absence of salt.

tribute to the total polymer in two ways: (a) by polymerizing simultaneously but independently when  $\alpha\beta_S$  is polymerizing and (b) by co-polymerization. The former seems unlikely because ~70% of the starting material had been digested as



**FIG. 6. The critical concentration for peak I polymer.** ●, purified tubulin was adjusted to the protein concentrations indicated in assembly buffer + GTP (1 mM). Subtilisin was added to 10 µg/ml and assembly was monitored by turbidity at 350 nm. The plateau value of absorbance is plotted versus the protein concentration. ○, purified tubulin was adjusted to 3 mg/ml as above and subtilisin was added to 10 µg/ml. After plateau absorbance was achieved, 1% v/v PMSF (1% w/v in dimethyl sulfoxide) was added to stop digestion. The sample was then diluted with assembly buffer to the indicated protein concentrations and turbidity was measured.

gleaned from SDS gels; hence the residual tubulin was well below its critical concentration of ~2 mg/ml. Similar results were obtained when the starting total tubulin concentration was 1 mg/ml. Moreover, the rapid onset of polymerization is inconsistent with microtubule assembly under these conditions. The possibility of co-polymerization cannot be ruled out and is discussed below.

#### Tubulin S ( $\alpha_S\beta_S$ )

When cleavage by subtilisin is allowed to proceed beyond the stage of the  $\alpha\beta_S$  (peak I) polymer, a fall in optical density is observed. This is followed by the formation of a second optical density peak that invariably attains a value that is approximately 2–4 times that of the first peak (Fig. 1). Fig. 2A reveals that this change coincides with the cleavage of the  $\alpha$  subunit to  $\alpha_S$  which is reduced in molecular mass by ~2 kDa on SDS gels. This cleavage is reflected in a further marked reduction of negative charge when electrophoresis is carried out in nondenaturing conditions (Fig. 2B). The resulting product, termed tubulin S, of composition  $\alpha_S\beta_S$  (Fig. 3), polymerizes with characteristics that are strikingly different from the  $\alpha\beta_S$  (peak I) polymerization and more nearly resembles assembly of intact tubulin, although there are differences. These properties are detailed below.

**Cold Sensitivity**—Although the  $\alpha\beta_S$  polymer is cold-insensitive, this property is lost upon further digestion, as shown in Fig. 7. When prewarmed tubulin and subtilisin are mixed at zero time, the typical optical density profile already depicted in Fig. 1 is observed. Depolymerization by cold followed by repolymerization gives absorbance equal to the second peak only. Note that depolymerization of peak II polymer progresses to near base-line values and is well below the level of peak I. Repolymerization of peak II material has never yielded the peak I intermediate seen initially. We believe this to be due to the irreversible conversion of the  $\alpha$  subunit to  $\alpha_S$  such that there is no longer an opportunity to form the  $\alpha\beta_S$  intermediate.

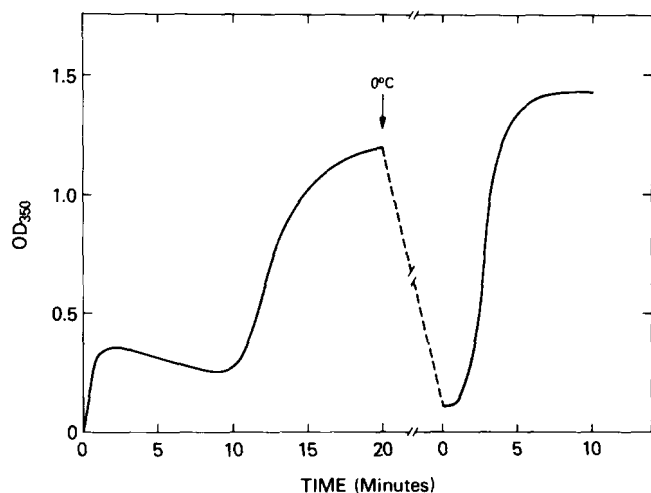


FIG. 7. **The effect of cold treatment on the polymer.** Turbidimetric scan of subtilisin-induced polymerization. Tubulin (1.5 mg/ml) in assembly buffer containing 1 mM GTP was incubated with subtilisin (1% w/w) at 37 °C. At the indicated time, the sample was placed on ice for 5 min and returned to the spectrophotometer, and the temperature was raised to 37 °C again.

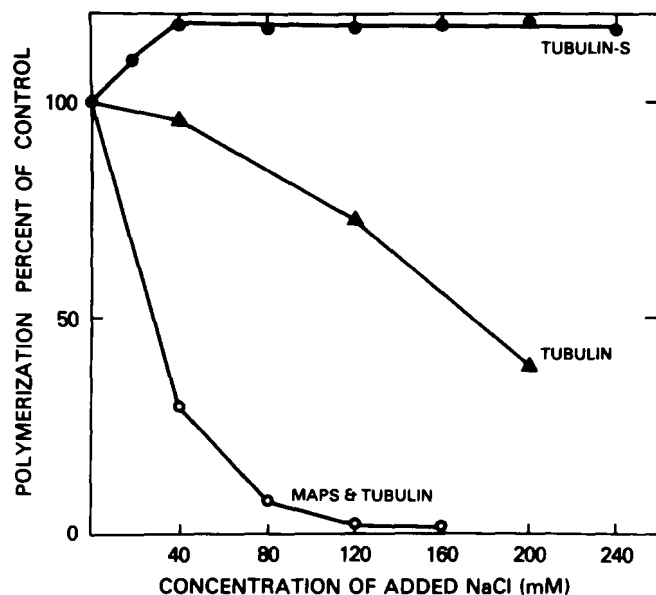


FIG. 8. **The effect of NaCl on polymerization.** NaCl was added to the reaction mixture before incubation at 37 °C and then polymerization was initiated with 1 mM GTP. Protein concentrations were 1.3 mg/ml, 3 mg/ml, and 1.04 mg/ml, respectively, for tubulin + microtubule-associated proteins, tubulin, and tubulin S polymerization. Tubulin S was prepared by incubation of tubulin (3.12 mg/ml) with subtilisin (1% w/w) at 30 °C for 45 min, PMSF was added, and the solution was kept at 0 °C for 30 min before use in experiments. In all cases, polymerization without the addition of NaCl was taken as 100%.

**Sensitivity to Depolymerizing Agents**—We have shown previously (15) that the tubulin S polymer is completely disassembled by 5 mM GDP or 50  $\mu$ M podophyllotoxin. We also noted a GTP requirement for polymerization of tubulin S (15) which is in striking contrast to the lack of such a requirement for the polymer formed from the intermediate,  $\alpha\beta_s$  (Fig. 5A). While this requirement resembles that exhibited by intact tubulin, other depolymerizing agents demonstrate significant differences between tubulin and tubulin S, *e.g.* in the sensitivity of polymerization to NaCl (Fig. 8). The polymerization of tubulin or tubulin + microtubule-associated proteins is sen-

sitive to rather low concentrations of NaCl and is strongly inhibited by 100 mM NaCl (28). In contrast, tubulin S polymerization is slightly enhanced by low concentrations of NaCl and even 240 mM NaCl does not cause inhibition. In this experiment, the protein concentration is different for each of the three cases (tubulin, tubulin + microtubule-associated proteins, tubulin S) because the critical concentration for polymerization is different in each case. The experiment addresses the polymerization of a standard amount of protein above its critical concentration. In all cases, the concentration of NaCl is nearly  $10^3$  times higher than that of the protein. These results suggest that the salt sensitivity of tubulin and tubulin + microtubule-associated protein polymerization involves the C termini of tubulin. In addition, these results provide a simple means of distinguishing polymers of tubulin from polymers of tubulin S.

An additional difference between tubulin and tubulin S is the markedly diminished  $Ca^{2+}$  sensitivity exhibited by the proteolyzed product. As shown in Fig. 9A, when 1 mM  $Ca^{2+}$  is added to microtubules made from normal tubulin, very rapid depolymerization is seen, as has been reported by many others previously. When the same concentration of  $Ca^{2+}$  is added to polymer formed from tubulin S, virtually no depolymerization occurs. The small drop appears to be due largely to dilution since it can be simulated by equal volumes of  $Ca^{2+}$ -free buffer. When  $Ca^{2+}$  is added at the beginning of polymerization, assembly occurs at an equal rate and to an equal extent as in the absence of the cation (Fig. 9B). Under these conditions, native tubulin is totally prevented from polymerizing.

#### Mixed Polymer Formation

The reduced  $Ca^{2+}$  sensitivity of tubulin S, its low critical concentration (15), and the rapid repolymerization (Fig. 7) suggested the possibility that removal of the C termini promoted nucleation. Such nuclei might be able to organize polymerization of native tubulin. It is well known that microtubules from various species will nucleate heterologous tubulin (30). It is also known that tubulin from species as divergent as yeast and mammalian brain will co-polymerize (31, 32). We, therefore, investigated the interaction of native rat brain tubulin with tubulin S derived from it. In the experiments of Fig. 10, tubulin and tubulin S were used at concentrations at which the individual proteins do not polymerize by themselves. However, when the two proteins are combined, polymerization does take place. The polymer which is formed is cold-sensitive, as are polymers formed with tubulin or tubulin S alone at higher concentrations (15). The mixed polymer also shows an intermediate salt sensitivity that is not characteristic of either protein alone. The presence of 240 mM NaCl causes a 40% loss of optical density (data not shown). If the polymer were tubulin, this concentration of salt would result in 100% loss of turbidity while no effect would be observed on a polymer of tubulin S (Fig. 8). Thus, both proteins appear to participate in the polymerization.

The interaction of both proteins is also shown in the data of Fig. 11. In these experiments, the apparent critical concentration for polymerization was determined for mixtures of tubulin and tubulin S. At a mole fraction of  $<0.1$ , tubulin S reduces the apparent critical concentration of the mixture by more than an order of magnitude. That both proteins in these mixtures polymerize is indicated by the following reasoning. The observed apparent critical concentration of a mixture of 90% tubulin, 10% tubulin S is approximately 0.1 mg/ml. If only the tubulin S were polymerizing, the expected critical concentration of the mixture would be calculated as the critical concentration of pure tubulin S (0.04 mg/ml) divided

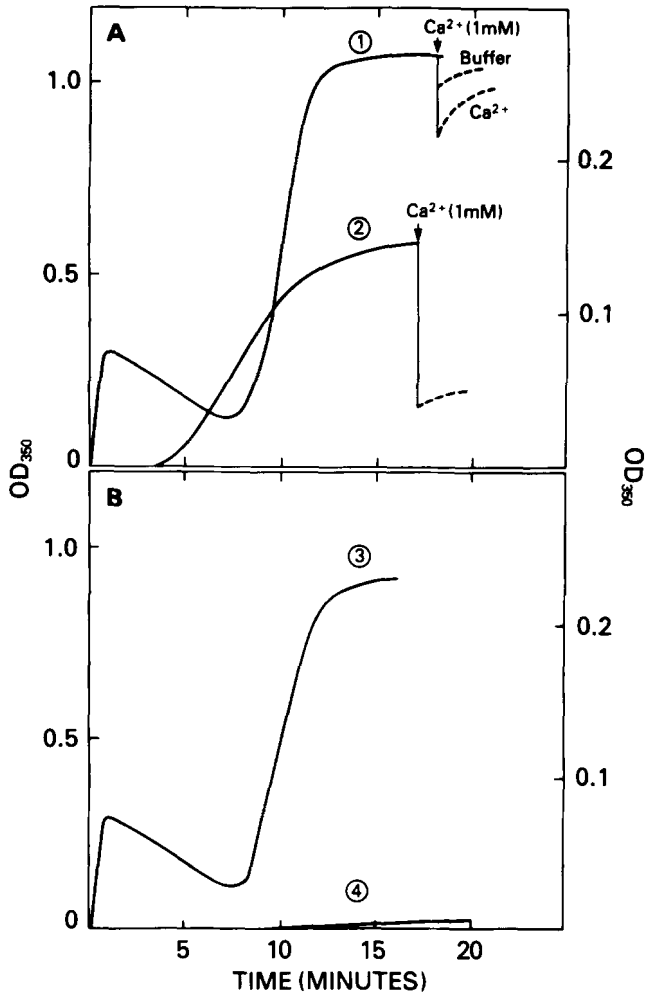


FIG. 9. The effect of calcium on polymerization. A: curve 1, turbidimetric scan at 350 nm of subtilisin-induced polymerization. Immediately before the scan was started, subtilisin (1% w/w) was added to the reaction mixture containing tubulin (1.04 mg/ml) and GTP. Prewarmed  $\text{CaCl}_2$  (1 mM final) was added at the indicated time. A control experiment was done by addition of an identical volume of prewarmed buffer. Curve 2, turbidimetric scan of tubulin (3 mg/ml),  $\text{CaCl}_2$  (1 mM final) was added at the indicated time and incubated further. B: curve 3, turbidimetric scan of subtilisin-induced polymerization. Tubulin (1.04 mg/ml) was pretreated with 1 mM  $\text{CaCl}_2$  before subtilisin (1% w/w) was added and the scan was performed at 37 °C. Curve 4, turbidimetric scan of tubulin (3 mg/ml) pretreatment with 1 mM  $\text{CaCl}_2$  before the polymerization was initiated. Note scale changes for  $\text{OD}_{350}$ . Curves 1 and 3 are plotted to the left abscissa; curves 2 and 4 to the right abscissa.

by the fraction of total protein present as tubulin S (0.1) or 0.4 mg of mixture/ml. Likewise, if only tubulin were polymerizing, the apparent critical concentration for the mixture would be 2.1/0.9 or 2.3 mg of mixture/ml. That the value obtained is different from either of these values or a simple average of these values indicates a polymerization reaction different from the polymerization of either pure tubulin or tubulin S. The important point is that small amounts of tubulin S have a remarkable lowering effect on the critical concentration of native tubulin. This is similar to the effect on the critical concentration of tubulin produced by microtubule-associated proteins.

#### DISCUSSION

Despite the considerable degree of homology between the sequences of  $\alpha$  and  $\beta$  tubulin subunits (33, 34), proteolysis by

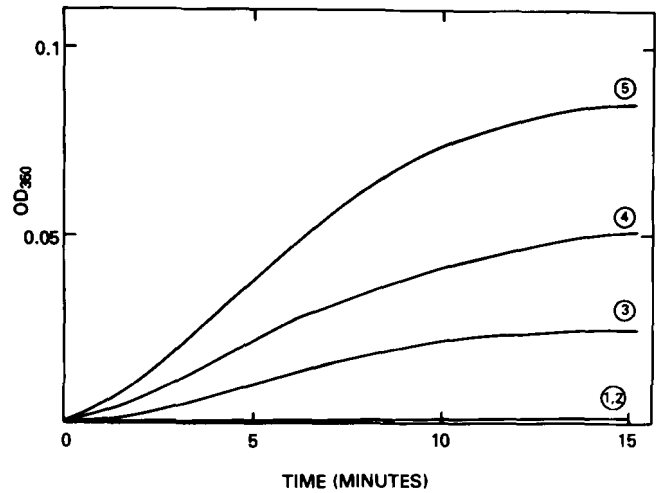


FIG. 10. The effect of the addition of tubulin S on the polymerization of tubulin. Samples of tubulin (0.8 mg/ml) in 50 mM Mes (pH 6.7), 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, and 1 mM GTP were polymerized with increasing concentrations of tubulin S. Tubulin concentration was 0.8 mg/ml in all cases; the concentrations of tubulin S are 0.03, 0.06, and 0.12 mg/ml in curves 3, 4, and 5, respectively. Control experiments with tubulin alone (0.8 mg/ml) and the highest concentration of tubulin S (0.12 mg/ml) are shown in curves 1 and 2, respectively. Tubulin S was made by incubating tubulin with subtilisin (1% w/w) in the presence of 1 mM GTP at 37 °C for 45 min, PMSF was added, and the solution was kept at 0 °C until use.

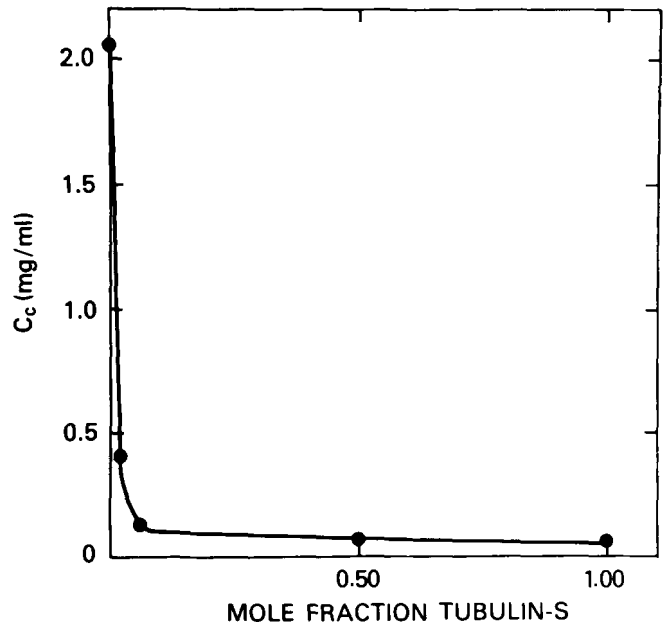


FIG. 11. Effect of addition of tubulin S on the critical concentration for tubulin polymerization. Tubulin S, prepared by the digestion of tubulin with subtilisin (1% w/w), was mixed with tubulin at different mole fractions. The critical concentration ( $C_c$ ) was then determined at each mole fraction by plotting plateau absorbance versus total protein concentration and extrapolating to zero absorbance. Plateau absorbances at three or four protein concentrations were determined for each mixture of tubulin and tubulin S.

subtilisin of these subunits is remarkably different. The  $\beta$  subunit is rapidly cleaved near its C terminus (15) even at low temperature, whereas the  $\alpha$  subunit is much more gradually cleaved at its C terminus and is not cleaved at low temperature (Figs. 2, 3, and 5). This marked discrepancy in the rates of hydrolysis leads to a transient state in which the hydrolytic mixture consists largely of unhydrolyzed  $\alpha$  subunit

and hydrolyzed  $\beta$  subunit ( $\beta_S$ ). Such a mixture of subunits can assemble into a hybrid polymer termed  $\alpha\beta_S$  or peak I. Progressive hydrolysis of  $\alpha$  to  $\alpha_S$  (Figs. 2 and 3) leads to a diminution or disappearance of this intermediate polymer (peak I) and final conversion to a different polymer (peak II) in which both subunits have lost their C termini (i.e.  $\alpha_S\beta_S$ ). As expected from the irreversible nature of the process required to form  $\alpha_S\beta_S$  (i.e. hydrolysis of  $\alpha$  to  $\alpha_S$ ), a second cycle of polymerization of tubulin S after cold depolymerization fails to go through the intermediate stage of peak I ( $\alpha\beta_S$ ) formation (Fig. 7).

At the pH values used in these experiments, the free carboxyl groups of the glutamyl and aspartyl residues of intact tubulin would be dissociated. Some of these are thought to be relatively mobile in the solvent on the basis of NMR data and have been assigned to the C termini (35). We postulate that the resulting charge repulsion between the C termini, which carry many of the excess negative charges, could hinder the assembly process. Hence, removal of a portion of these negative charges may have a major effect in promoting polymerization. Within the limitations of these experiments, the apparent critical concentrations for tubulin,  $\alpha\beta_S$ , and tubulin S show a stepwise decrease as a function of the increasing removal of excess negative charge from the carboxyl termini. Other intermediate steps in charge removal would be useful to support this idea but to date we have not succeeded in producing them. Microtubule-associated proteins may accomplish a similar result by charge neutralization and thereby facilitate assembly. Such a suggestion has recently been made (25, 36).

The properties of the two polymers made from  $\alpha\beta_S$  and  $\alpha_S\beta_S$  exhibit some similarities as well as striking differences. These are summarized in Table I. In contrast to microtubules made from the starting material, both polymers formed after subtilisin cleavage are insensitive to high salt concentration and 1 mM  $\text{CaCl}_2$ . This suggests that a  $\text{Ca}^{2+}$ -sensitive site may reside in one or both carboxyl-terminal fragments. Polymers formed from the  $\alpha\beta_S$  intermediate are not sensitive to cold whereas the tubulin S polymers ( $\alpha_S\beta_S$ ) are exquisitely sensitive and depolymerize rapidly on ice to an extent of 80–90% of the total absorbance (Fig. 7). The differences also include a lack of sensitivity of  $\alpha\beta_S$  polymer to podophyllotoxin and the absence of a GTP requirement and/or GDP sensitivity for polymerization.

An additional difference resides in the type of polymer found in peak I and peak II. As shown in Fig. 4, the  $\alpha\beta_S$  polymers consist of rings that are much smaller than the linear polymers, sheets, and microtubules formed from tubulin S or from microtubules compared to native tubulin. The smaller size of the peak I polymer probably accounts for the decreased amount of scattering per unit protein and hence the lower amplitude of peak I.<sup>3</sup>

In the preceding paper we showed that the removal of the C termini from the  $\alpha$  and  $\beta$  subunits of tubulin by subtilisin led to a product (tubulin S) that polymerized more avidly than native tubulin but retained its GTP dependency, and

TABLE I

Properties of peak I ( $\alpha\beta_S$ ) and peak II ( $\alpha_S\beta_S$ ) polymers

| Properties   | Peak I                         | Peak II            |
|--|--------------------------------|--------------------|
| GTP requirement  | Not required                   | Required           |
| Temperature effect (0 °C)                              | Insensitive                    | Sensitive          |
| Calcium effect on assembly (1 mM)                      | <i>Insensitive<sup>a</sup></i> | <i>Insensitive</i> |
| Salt effect (240 mM NaCl)                              | <i>Insensitive</i>             | <i>Insensitive</i> |
| Podophyllotoxin effect on assembly (50 $\mu\text{M}$ ) | Insensitive                    | Sensitive          |
| GDP effect on assembly (50 $\mu\text{M}$ )             | Insensitive                    | Sensitive          |
| Critical concentration (mg/ml)                         | 0.4 mg/ml                      | 0.04 mg/ml         |

<sup>a</sup> Italics indicate similarities in properties.

cold and podophyllotoxin sensitivity (15). Because the critical concentration was markedly lowered, it was important to know whether tubulin S could induce polymerization of native tubulin or, alternatively, whether both forms of tubulin would co-polymerize. Figs. 10 and 11 show that polymer formation occurs readily in mixtures of the two tubulins at concentrations of each which are incapable of polymerizing. The polymers so formed contain both forms of tubulin. The fact that the presence of a few per cent of tubulin S may promote polymerization at concentrations of total tubulin that are below the critical concentration for native tubulin, suggests an interesting possibility for *in vivo* proteolysis in polymerization under otherwise unfavorable polymerization conditions. Effective amounts of tubulin S might be very difficult to detect in the usual warm/cold-cycled preparations and special methods will have to be devised to test this hypothesis.

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<sup>3</sup> The formation of rings by the singly cleaved hybrid dimers,  $\alpha\beta_S$ , and not by the doubly cleaved  $\alpha_S\beta_S$  dimer, raises the possibility that formation of rings during cold depolymerization of intact microtubule may be due to a transient state of dimer conformation. In this view, depolymerization is a stepwise process, moving from the polymer state, in which the C termini of both subunits are “neutralized” (as by e.g. microtubule-associated proteins or intramolecular charge interaction) to the free dimer state, in which both are “free.” The intermediate state, where the C terminus of one subunit remains “neutralized” while the other is “free,” would be the state with ring-forming potential.



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