Manning, G. S. (1978) *Q. Rev. Biophys. 11*, 179–246.
McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.
Newport, J. W., Lonberg, N., Kowalczykowski, S. C. & von Hippel, P. H. (1981) *J. Mol. Biol. 145*, 105–121.
Park, C. S., Wu, F. Y. H., and Wu, C. W. (1982) *J. Biol. Chem. 257*, 6950–6956.
Peterman, B., & Wu, C. W. (1978) *Biochemistry 18*, 3889–3892.
Pörschke, D., & Rauh, H. (1983) *Biochemistry 22*, 4737–4745.
Record, M. T., Lohman, T. M., & de Haseth, P. L. (1976) *J. Mol. Biol. 107*, 145–158.
Record, M. T., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.
Riggs, A. D., Bourgeois, S., & Cohn, M. (1970) *J. Mol. Biol. 53*, 401–417.
Roe, J., Burgess, R. R., & Record, M. T., Jr. (1984) *J. Mol. Biol. 176*, 495–522.
Russel, M. L., Gold, L., Morrissett, H., & O'Farrell, P. Z. (1976) *J. Biol. Chem. 251*, 7263–7270.
Shiao, D. D. F., & Sturtevant, J. M. (1976) *Biopolymers 15*, 1201–1211.
von Hippel, P. H., Kowalczykowski, S. C., Lonberg, N., Newport, J. W., & Paul, L. S. (1983) *J. Mol. Biol. 162*, 795–818.
Winter, R. B., Berg, O. G., & von Hippel, P. H. (1981) *Biochemistry 20*, 6961–6977.

Controlled Proteolysis of Tubulin by Subtilisin: Localization of the Site for MAP_2 Interaction†

Luis Serrano,* Jesús Avila, and Ricardo B. Maccioni

ABSTRACT: The treatment of tubulin with subtilisin resulted in a significant decrease in the ability of tubulin to assemble. The addition of taxol reduced the effect of subtilisin on the assembly of digested protein. Limited proteolysis of tubulin by subtilisin affected simultaneously both α- and β-subunits, and it resulted in the appearance of two major cleavage fragments (32 and 20 kilodaltons) or an alternative pattern yielding two fragments (48 and 4 kilodaltons). The smallest peptide (4 kilodaltons) and also the 20-kilodalton fragment are localized in the C-terminal region of the tubulin α-subunit. Digested tubulin can assemble into sheet-shaped polymers, which cannot incorporate MAP_2. On the other hand, the isolated C-terminal fragments can bind to MAP_2. These results suggest that the carboxyl-terminal domain of the tubulin molecule is the site for the MAP_2 interaction.

Tubulin, the major component of microtubules, can self-associate, bind nucleotides and specific drugs, or interact with other proteins associated with microtubules (MAPs).[1] It is very important to know the characteristics of the tubulin domains involved in these interaction processes. One approach has been done to study the functional role of specific tubulin residues in those associations (Maccioni et al., 1981a,b; Maccioni & Seeds, 1981, 1982; Mann et al., 1978; Palanivelu & Ludueña, 1982; Wadsworth & Sloboda, 1982; Mellado et al., 1980, 1982). Recently, limited proteolysis with trypsin and chymotrypsin (Brown & Erickson, 1983; Maccioni & Seeds, 1983) has proven very useful to fractionate the tubulin molecule into fragments and to analyze the tubulin structure as related to microtubule assembly. A similar approach has been previously used (Vallee & Borisy, 1977, 1978) to study the binding domain of the microtubule-associated protein MAP_2 to tubulin. Vallee (1980) has found that MAP_2 binds to tubulin through its cationic terminal region. However, nothing is known about the tubulin domain bound to MAP_2. For the present report, we have done a limited cleavage at specific sites of the tubulin molecule with subtilisin to study the structural domains of tubulin involved in the interaction with MAP_2. Our results indicate that the carboxyl-terminal region of tubulin is involved in the interaction with MAP_2.

Materials and Methods

Purification of Tubulin. Tubulin from pig brain was prepared by temperature-dependent cycles of assembly–disassembly following the procedure of Shelanski et al. (1973) and stored as pellets at −70 °C. Microtubule pellets were resuspended in 0.1 M MES (pH 6.4), 2 mM EGTA, and 0.5 mM $MgCl_2$ (buffer A) prior to their use, and a third cycle of assembly–disassembly was performed. Tubulin depleted of MAPS was obtained by phosphocellulose chromatography (PC-tubulin) as described by Weingarten et al. (1975). The microtubule-associated protein MAP_2 was isolated according to the procedure of Sandoval & Weber (1980).

The protein concentration of microtubule protein was determined by the procedure of Lowry et al. (1951) and the concentration of PC-tubulin by $A_{280} = 1.15$ mg·mL^{-1} (Appu Rao et al., 1978).

Radioactive Labeling of Tubulin. The ^{125}I-iodinated tubulin used in some experiments was prepared according to the procedure of Carlier et al. (1980). The radiolabeling resulted

† From the Centro de Biologiá Molecular (CSIC-UAM), Facultad de Ciencias, Universidad Autonoma de Madrid, Canto Blanco, Madrid-34, Spain. *Received February 3, 1984.* This work was supported in part by the Fund of Comisión Asesora and the Programa de Cooperación Internacional, Ministerio de Educación, Spain.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTP, guanosine 5′-triphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; C-terminal, carboxyl terminal; N-terminal, amino terminal; $NaDodSO_4$, sodium dodecyl sulfate; MAPs, microtubule-associated proteins; PMSF, phenylmethanesulfonyl fluoride; PC-tubulin, phosphocellulose-purified tubulin.

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in ^{125}I-PC-tubulin with a specific activity of 20 μCi/mg of protein.

In some experiments, tubulin was labeled in the carboxyl terminus of the α-subunits with [^{14}C]tyrosine by using the procedure of Arce et al. (1975). The [^{14}C]tyrosyltubulin was obtained after two polymerization cycles with a specific radioactivity of 40–170 cpm/μg.

Proteolytic Digestions. The digestion of tubulin samples with subtilisin (Sigma) was performed by incubation at 30 °C for 30 min unless otherwise indicated, at the concentration indicated in each experiment. Aliquots from the incubation mixture was obtained, and after addition of 2 mM PMSF and appropriate dilutions, the samples were assayed for assembly activity or treated for acrylamide–NaDodSO$_4$ electrophoresis. Control samples were incubated under identical conditions but without the proteolytic enzyme.

Assembly Assay. Uncleaved tubulin, subtilisin-digested tubulin, and microtubule protein were assembled by incubation at 37 °C for 30 min in 0.1 M MES, pH 6.4, 0.5 mM MgCl, and 2 mM EGTA and by the addition of 1 mM GTP. For tubulin depleted of MAPs, the addition of 10 μM taxol, 10% (v/v) Me_2SO, or 0.5% (w/w) MAP_2 was required for assembly. In certain experiments indicated in the text, some of these compounds were also added for the assembly of unfractionated microtubule protein.

The assembled protein was isolated after centrifugation in an airfuge (Beckman) for 10 min at 96 000 rpm at room temperature. The protein present in the sedimented pellets was quantified either by using the method of Lowry et al. (1951) or by measuring the radioactivity associated with the pellets when either ^{125}I-tubulin or ^{14}C-tyrosinated tubulin was used in the experiments. Samples assayed under identical conditions except for the omission of GTP were used as blanks.

Copolymerization analysis using ^{125}I-MAP_2 was done by mixing 3 μg of MAP_2 (8000 cpm) with increasing amounts of the purified C-terminal fragment of tubulin subunits (4-kilodalton fragment) (from 0 to 40 μg) in buffer A plus bovine serum albumin (1 mg/mL). The mixture was incubated for 5 min at room temperature followed by an incubation for 60 min at 4 °C in the presence of 1% formaldehyde for cross-linking the 4K molecular weight fragment to MAP_2. The reaction was stopped by adding glycine in a proportion of 2:1 (w/w) with respect to formaldehyde followed by dialysis. Copolymerization was performed by adding the previous mixture to a solution of microtubule protein (2 mg/mL) containing 1 mM GTP. The whole mixture was incubated for 30 min at 30 °C, and the assembled protein was isolated by centrifugation as previously indicated.

Binding of the carboxy-terminal tubulin fragment to MAP_2 was tested by incubation of 5 μg of C-terminal peptide with 30 μg of MAP_2 for 15 min at room temperature, followed by fractionation of the complex by chromatography on a Sepharose 6B column (0.8 × 18 cm). Fractions of 0.35 mL were collected, and the radioactivity present in them was measured.

Electrophoresis. Tubulin samples obtained during the proteolysis experiments were adjusted to 1% NaDodSO$_4$, 1 M glycerol 2% (v/v) 2-mercaptoethanol, and 0.001% bromophenol blue, boiled, and analyzed by electrophoresis in polyacrylamide–NaDodSO$_4$ slab gels (7–11%) by the procedure of Laemmli (1970) with a constant current of 20 mA/gel. Gels were stained according the method of Fairbanks et al. (1971) using Coomassie blue. The resolve in the same gel polypeptides with molecular weights from approximately 2500 to 90 000, the conditions described by Anderson et al. (1983) were followed.

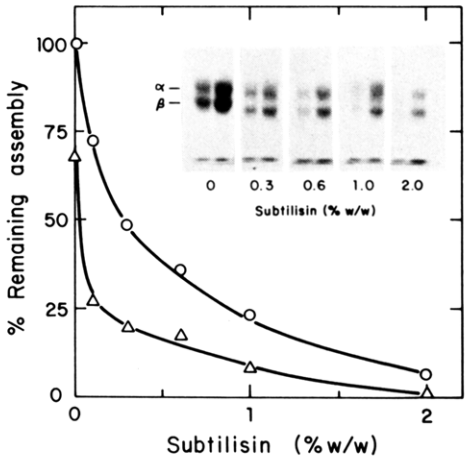

FIGURE 1: Inactivation of tubulin assembly by digestion with increasing concentrations of subtilisin (% w/w with respect to tubulin). Microtubule protein samples (2 mg/mL), obtained by three cycles of assembly–disassembly, were incubated at 30 °C for 30 min with increasing concentrations of subtilisin. The digestion was terminated by addition of 2 mM PMSF and decreasing the temperature to 4 °C. Two different aliquots of 125 μL were obtained from each sample and assayed for microtubule assembly. The amount of protein assembled in the presence (○) or absence (△) or 10 μM taxol is shown. Another set of 125-μL aliquots were incubated under assembly conditions in either the presence (right lanes) or the absence (left lanes) of 10 μM taxol and sedimented, and the pellets were subjected to electrophoresis in a 7% acrylamide slab gel (inset).

In experiments in which ^{125}I- or ^{14}C-tyrosinated tubulin was used, some strips of the gels were sliced into 2-mm pieces to determine the radioactivity associated with tubulin and cleavage products of the molecule. In the case of ^{14}C-tyrosinated tubulin, the gel slices were dissolved by incubation for 24 h at 60 °C with 0.5 mL of 99% H_2O_2 (30% v/v) and 1% NH_4OH, and the radioactivity was measured after the addition of 10 times the volume of the sample of Triton–toluene scintillation cocktail.

Electron Microscopy. The assembly products of digested or undigested tubulin samples were analyzed in a JEOL Model 100-B electron microscope after 1:10 dilution of assembled samples in 0.5% glutaraldehyde and staining in 1% uranyl acetate.

Results

Limited Proteolysis of Microtubule Protein by Subtilisin. Limited proteolysis of 3 times cycled microtubular protein with the proteolytic enzyme sibtilisin resulted in a marked decrease of its assembly ability. Microtubular protein samples were digested at 30 °C for 30 min with increasing concentrations of subtilisin (0–2% w/w to tubulin), and the assembly was assayed in either the presence or the absence of 10 μM taxol. The increase of subtilisin concentration resulted in a decrease of assembly with an almost complete inactivation at 2% subtilisin (Figure 1).

However, when assembly was assayed in the presence of taxol, a lesser degree of inactivation was observed. The effect of taxol was most noticeable when low concentrations of subtilisin were used, i.e., before more extensive cleavage of tubulin occurred. Clearly, the assembly inactivation is related to a significant decrease of the amount of tubulin in the sedimented polymer when assembly was performed in either the presence or the absence of taxol as shown by electrophoresis of subtilisin-treated samples (inset of Figure 1).

The effects of subtilisin on the taxol-promoted assembly of tubulin depleted of microtubule-associated proteins (MAPs) were also examined. Figure 2 shows the time-dependent in-

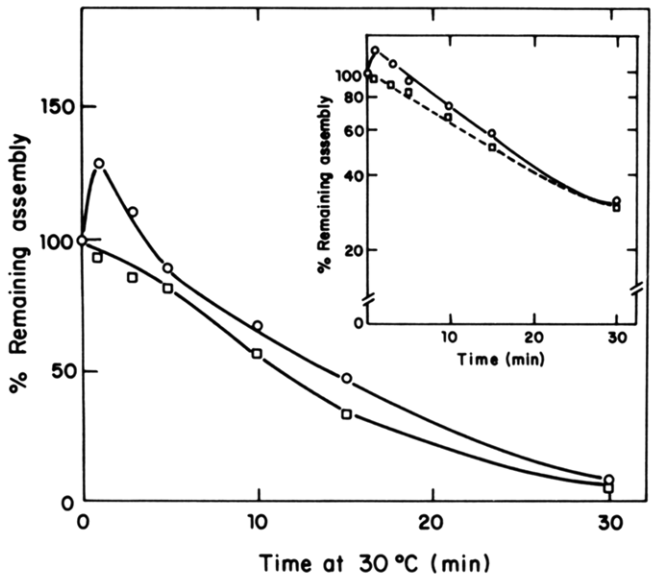

FIGURE 2: Time-dependent inactivation of PC-tubulin and microtubule protein assembly by digestion with subtilisin. One-milliliter samples of either PC-tubulin (2 mg/mL) (□) or 3 times cycled microtubule (○) protein (2 mg/mL) were mixed separately with 70 μL of ^{125}I-PC-tubulin; the final specific activity of each mixture was 2×10^3 cpm/μg. The mixtures were digested at 30 °C with 1.2% (w/w) subtilisin, 0.14 mL of each sample was obtained at the time intervals indicated, and the microtubule assembly was assayed by sedimentation and determination of the radioactivity of the ^{125}I-tubulin in the pellets or by protein quantitation. Assembly was assayed in the presence of 10 μM taxol. The inset shows a semilogarithmic plot of the inactivation kinetics; 100% indicates the assembly in the absence of added protease.

activation of both microtubular proteins and phosphocellulose-treated tubulin (PC-tubulin) by proteolysis at 30 °C with 1.2% subtilisin. A slightly higher time-dependent inactivation was observed for PC-tubulin proteolysis. Figure 2 also indicates a slight activation of assembly after a short exposure to subtilisin of the microtubule protein preparation. This activation was consistently observed at different concentrations of subtilisin.

Characterization of the Polymers Assembled in the Presence of Subtilisin-Digested Tubulin. As indicated above, limited proteolysis with subtilisin decreases but does not completely eliminate polymerization of tubulin. It suggests that digested tubulin could be incorporated into the polymers. We have characterized the polymers assembled in the presence of subtilisin-digested tubulin by electron microscopy and by gel electrophoresis to test whether some of the fragments of the tubulin could be found associated with polymers and whether such polymers are microtubules.

Electron microscopy observations of the taxol-promoted assembly products from digested microtubular protein revealed the presence of abundant sheets, twisted ribbons, and less organized structures of protofilaments, but not microtubules. Normal microtubules were observed in the assembled polymers obtained from the undigested tubulin control (Figure 3). The same results were obtained when Me_2SO was used instead of taxol to promote the polymerization of the digested tubulin (Figure 3C). The aberrant polymer forms found are probably not due to the lower concentration of assembly-competent tubulin present after protease treatment because results similar to those of Figure 3 were obtained when the microtubule protein was assembled at 1, 2, or 3 mg/mL.

Characterization of the Fragments from Tubulin Digested by Subtilisin. The cleaved tubulin molecule assembled into polymers was analyzed by electrophoresis. Figure 4 shows that

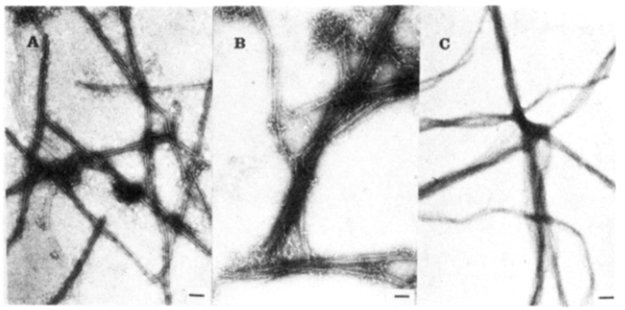

FIGURE 3: Electron microscopy of the assembly products from tubulin digested with subtilisin and from the undigested tubulin control. (A) Microtubules assembled from 3 times cycled tubulin incubated in the absence of subtilisin. (B) Assembly products from tubulin incubated at 30 °C for 15 min with 1.2% subtilisin. Samples in panels A and B were assembled in the presence of 10 μM taxol. (C) Assembly products from tubulin incubated at 30 °C for 15 min with 1.2% subtilisin. Samples were assembled in the presence of Me_2SO. The bars represent 0.1 μm.

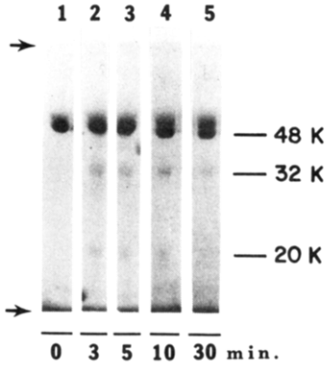

FIGURE 4: Polyacrylamide–NaDodSO$_4$ slab gel of the assembled polymer from PC-tubulin digested with subtilisin for different time intervals. A PC-tubulin sample (2 mg/mL) was incubated at 30 °C in the presence of 1.2% subtilisin, and aliquots were obtained at 0 min and at increasing time intervals. The incubation was terminated as indicated under Materials and Methods, the samples were assembled and centrifuged in an airfuge, and the protein in the pellets (50 μg) was subjected to electrophoresis in a 11% acrylamide–NaDodSO$_4$ gel. The positions corresponding to the M_r 48K, 32K, and 20K peptides are indicated.

the major digestion products of tubulin were two polypeptides of M_r 32K (Ps-1) and 20K (Ps-2). At longer times of digestion or by using different concentrations of subtilisin, two fragments appear with electrophoretic mobilities slightly faster than those of the tubulin α- and β-subunits and with molecular weights of 48–49K (see inset of Figure 1 and Figure 4). The above results suggest the release of a small peptide of about M_r 4K. This peptide was detected in electrophoresis (see below, Figure 6).

The changes in the amount of α- and β-subunits as well as the amount of M_r 32K and 30K fragments incorporated into the polymers were analyzed by using ^{125}I-tubulin depleted of MAP_2. The data of Figure 5 show that both α- and β-subunits were cleaved with similar kinetics and that the proportion of polypeptides Ps-1 and Ps-2 incorporated into polymers also decreased at early times of subtilisin digestion. Simultaneously, the ability of assembly decreased during the incubation with the enzyme. However, when the amount of tubulin fragments was analyzed (data not shown) in the total digested tubulin sample prior to assembly, it was found that Ps-2 increased during the first minutes of subtilisin digestion and thereafter decreased with the time of incubation while Ps-1 increased during proteolysis, indicating that Ps-1 is more resistant than Ps-2 to a further proteolysis by subtilisin.

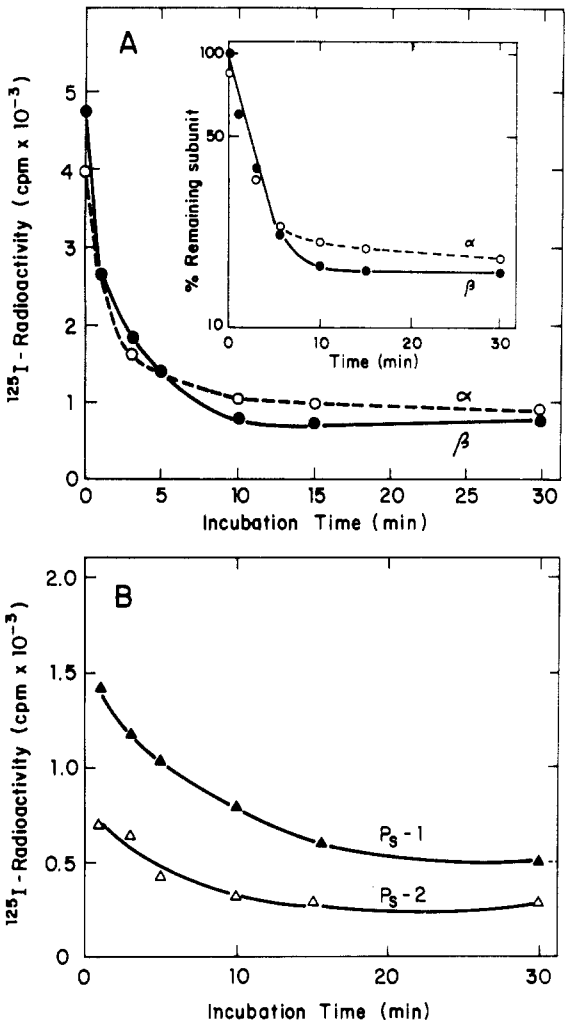

FIGURE 5: Proteolysis kinetics of PC-tubulin digested with subtilisin. ^{125}I-Labeled tubulin (2 mg/ml)(2 × 10^3 cpm/μg) was digested with 1.2% (w/w) subtilisin in a final volume of 1 mL under conditions described in Figure 2. Fractions of 0.14 mL of each sample were obtained at the time intervals indicated, adjusted to 1 mM GTP, 1.2 mM $MgCl_2$, 0.5 mM EGTA, and 10 μM taxol, assembled, and centrifuged in the airfuge. The pellets were resuspended in the electrophoresis buffer, subjected to electrophoresis in 11% acrylamide gels, and stained with 0.05% Coomassie blue. The stained tubulin subunits (A) and the cleavage products of tubulin, Ps-1, and Ps-2 (B), were cut from the gel, and the ^{125}I radioactivity was determined.

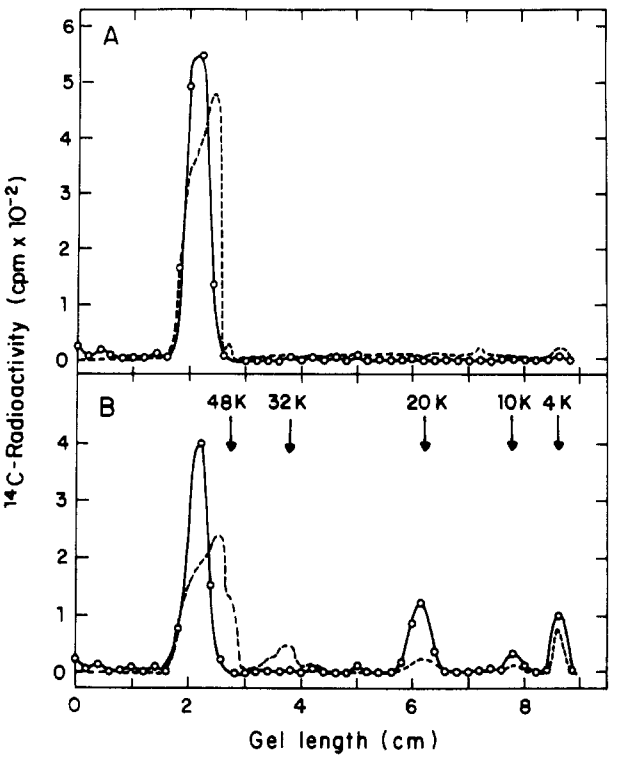

FIGURE 6: Electrophoresis of [^{14}C]tyrosyltubulin incubated in the presence and absence of subtilisin. Sample of 0.8 mL of PC-tubulin (2.5 mg/mL) was mixed with 0.2 mL of [^{14}C]tyrosyl tubulin (1 mg/mL) (120 cpm/μg). Half of the sample was incubated in the presence of 1.2% w/w subtilisin and the other half in the absence of the enzyme for 15 min. The reaction was stopped by the addition of electrophoresis sample buffer containing 2 mM PMSF and by boiling. Samples were electrophoresed in 11% acrylamide gels, the gels were sliced in 2-mm pieces, and the radioactivity was counted as indicated under Materials and Methods. (A) Radioactivity patterns of nondigested tubulin (—). (B) Radioactivity patterns of the PC-tubulin sample digested for 15 min with subtilisin (—). The dashed lines represent the densitometric profiles of the Coomassie-stained electrophoretic gel, either in (A) or in (B). The arrows indicate the mobility of the 48K, 32K, 20K, and 4K fragments.

To localize the tubulin fragment containing the carboxyl-terminal region, ^{14}C-tyrosinated tubulin was incubated in either the presence or the absence of subtilisin, and the resulting peptides were fractionated by electrophoresis. Figure 6 shows that in addition to α-tubulin, ^{14}C radioactivity was mainly associated with the M_r 20K fragment (Ps-2) and with a small peptide of about M_r 4K. Also, there was some radioactivity associated with a peptide of about M_r 10K, probably a proteolytic peptide of the M_r 20K fragment.

Interaction of MAP_2 with Tubulin. It has been indicated by experiments involving modification of thiol groups (Ludueña et al., 1982) or by cross-linking analysis (J. L. Carrascosa, J. C. Diez, and J. Avila, unpublished results) that MAP_2 can interact with both α- and β-subunits, possibly through the interaction with a sequence present in both subunits due to the close relationship between the sequence of both polypeptides, especially in some domains of the molecule (Ponstingl et al., 1981; Krauhs et al., 1981). These domains could be involved in the tubulin–MAP_2 interaction.

On the basis of the tubulin cleavage data, it was judged of interest to examine the capacity of the digested preparations to incorporate MAP_2. Figure 7 (panels I and II) shows that MAP_2 failed to be incorporated into the digested PC-tubulin polymers assembled as indicated under Materials and Methods. Lanes b of Figure 7 show the lack of association of MAP_2 with subtilisin-digested tubulin, while MAP_2 was associated with the assembled polymer obtained from the nondigested tubulin control (lanes d). The MAP_2 added to assemble the cleaved tubulin remained undigested in the supernatant (lanes a). One of the differences between the polymers of digested and undigested tubulin is that the first one lacks the carboxyl-terminal fragments (M_r 10K and 4K) as determined by electrophoresis in an experiment similar to that of Figure 6 (data now shown).

To test whether the carboxyl-terminal domain was the one which contains the sequence of tubulin that interacts with MAP_2, we have purified the carboxyl-terminal fragments from [^{14}C]tubulin by chromatography and rechromatography in a Sephadex G-50 column (Figure 8). When the pool of low molecular weight fragments isolated by the Sephadex G-50 column was subjected to electrophoresis, the ^{14}C-labeled fragments were mainly found in a broad band with an electrophoretic mobility corresponding to the M_r 4K fragment (≥80% of the peptide material) and two smaller peaks corresponding to M_r 10K and 20K fragments (data not shown). There was no contamination of either tubulin or higher molecular weight tubulin fragments. The M_r 48K and 32K fragments separated by electrophoresis of fractions from the

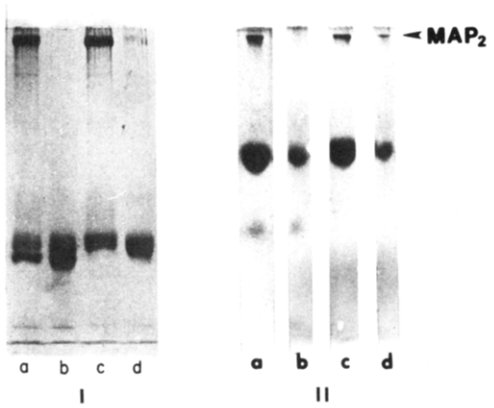

FIGURE 7: Incorporation of MAP_2 into microtubules obtained from digested and undigested PC-tubulin samples. Two samples of 0.1 mL of PC-tubulin (2 mg/mL) were incubated at 30 °C in either the presence or the absence of 1.2% subtilisin. After 30-min incubation, the digestion was terminated by addition of 2 mM PMSF and bringing the samples to 4 °C. Aliquots of 50 μL of MAP_2 (1.4 mg/mL) were added to both samples, allowed to assemble in the presence of 1 mM GTP, 10 μM taxol, and 2 mM PMSF, and centrifuged in the airfuge. Electrophoresis was performed in 7.5% acrylamide gels (I) or in 10% acrylamide (II). Experiments for panels I and II were performed 1 year apart, and several other experiments during the interim gave virtually the same results. The lanes in panels I and II indicate (a) supernatant after the assembly of PC-tubulin digested with subtilisin, (b) pellet after the assembly of PC-tubulin digested with subtilisin, (c) supernatant after the assembly of undigested PC-tubulin, and (d) pellet after the assembly of undigested PC-tubulin.

elution volume (V_0) of Sephadex G-50 chromatography (Figure 8) were not radiolabeled, in agreement with the information of Figure 6.

When the labeled fragments were incubated with MAP_2 and chromatographed on a Sepharose 6B column, we found that a fraction of the ^{14}C-labeled material eluted in the same volume as that of purified MAP_2. Also, a possible complex between MAP_2 and the carboxyl-terminal domain of tubulin eluted in the void volume of the column (Figure 9d). However, when a mixture of catalase plus albumin was added, instead of MAP_2, no association between these fragments and those proteins (Figure 9b) was found. The addition of purified 4K fragment to ^{125}I-MAP_2 prevents the further association of this protein to microtubules. Figure 10 shows a decrease in the incorporation of ^{125}I-MAP_2 to microtubules when increasing amounts of the C-terminal fragments of tubulin were added. These results suggest that the carboxyl-terminal end of α-tubulin is implicated in the interaction with MAP_2.

Discussion

The present studies of limited proteolysis of tubulin with subtilisin were undertaken to further understand the involvement of structural domains of the tubulin molecule in the assembly process. It has been shown that cleavage of microtubule protein is associated with a decrease of its assembly ability, as indicated by the assembly inactivation by digestion of PC-tubulin. In the microtubular protein, the decrease of the assembly activity could be due, in a first step, to a preferential proteolysis of a microtubule-associated protein such as MAP_2. These proteins decrease the critical concentration that tubulin needs to polymerize into microtubules (Sandoval & Weber, 1980), and their breakdown could result in a loss of microtubule assembly. In such conditions, addition of taxol, which also reduces the critical concentration for tubulin assembly, increases the extent of polymerization of digested tubulin, probably because it could somehow replace the function of MAP_2 or of other proteins with a similar function.

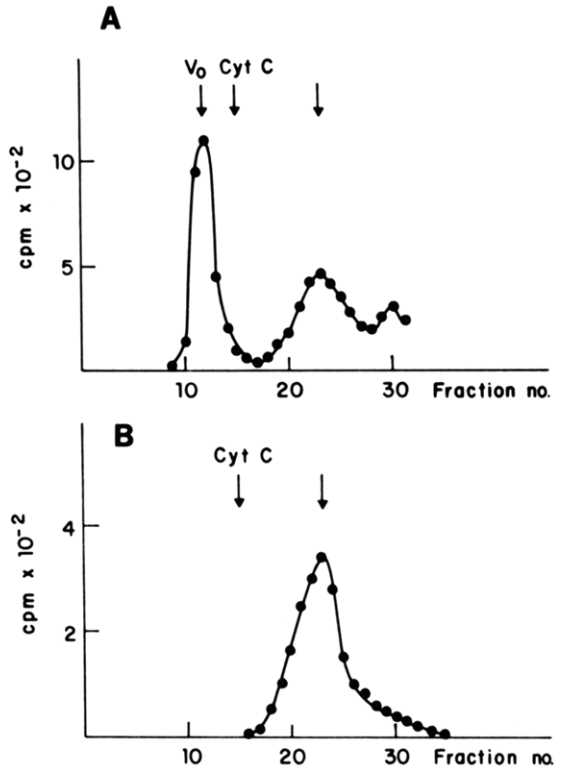

FIGURE 8: Purification of carboxyl-terminal fragments of tubulin. (A) Seventy microliters of ^{14}C-tyrosinated tubulin (5.8 mg/mL) (150 cpm/μg) was digested with 2% w/w subtilisin for 60 min at 30 °C. The digested sample was chromatographed on a Sephadex G-50 column (0.3 × 20 cm) equilibrated with 0.1 M MES, pH 6.2, 0.1 mM Mg^{2+}, and 2 mM EGTA buffer. Fractions of 0.2 mL were collected, and 10 μL of each fraction was used to measure the radioactivity as described under Materials and Methods. Twenty microliters of cytochrome *c* (Cyt *c*) (10 mg/mL) was chromatographed under the same conditions as a marker of the elution volume. (B) The fractions present in the peaks indicated by the arrows in (A) were rechromatographed under the same conditions as above, and the radioactivity was also measured. For preparative purposes, a column of Sephadex G-50 of 1 × 26 cm was used, and a similar peptide profile was obtained.

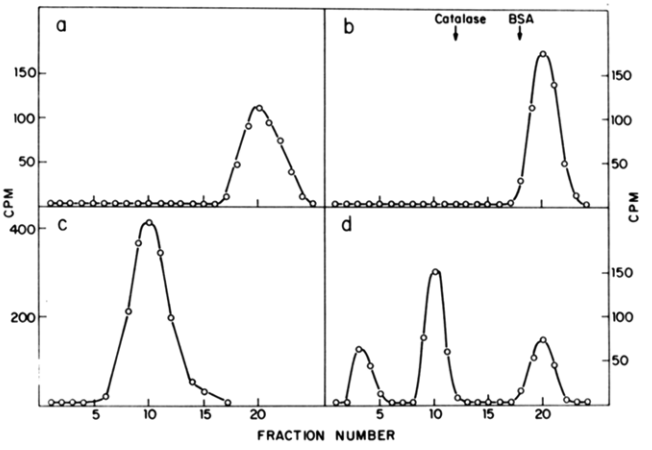

FIGURE 9: Binding of C-terminal tubulin fragments to MAP_2. Ten micrograms of ^{14}C-labeled fragments (40 cpm/μg) obtained as indicated in Figure 8 was chromatographed on a Sepharose 6B column (0.8 × 18 cm) equilibrated in buffer A alone (a) or in the presence of 500 μg of catalase and 400 μg of bovine serum albumin (b). The arrows indicated the elution position of such proteins. In (c) is indicated the elution position of ^{125}I-MAP_2, and (d) shows the result of mixing 10 μg of C-terminal fragment with 30 μg of MAP_2. Fractions of 0.35 mL were collected, and their radioactivities were measured. In this figure, tubulin dimer elutes between catalase and albumin peaks.

A further contribution to the decrease in the assembly activity of microtubule protein should result from the cleavage of

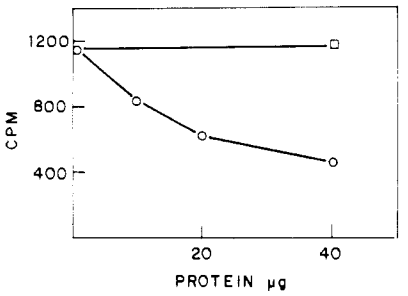

FIGURE 10: Effect of C-terminal fragments on the association of MAP_2 with microtubules. Three micrograms of ^{125}I-MAP_2 (8000 cpm) was mixed as indicated under Materials and Methods with increasing amounts of C-terminal tubulin fragment (O) or bovine serum albumin (□), and the resulting mixtures were added to 70 μL of a microtubule protein solution (2 mg/mL) in buffer A plus 1 mM GTP. The samples were incubated for 30 min at 30 °C, and the radioactivity associated with the assembled protein was determined in each case, together with the amount of polymerized protein. This amount was similar for every point indicated in the figure (~100 μg). The addition of bovine serum albumin does not affect microtubule assembly.

tubulin subunits by subtilisin.

The cleavage of tubulin by subtilisin yields two fragments of M_r 32K and 20K, corresponding to the N-terminus and C-terminus, respectively. However, at longer times of subtilisin digestion, tubulin subunits were also cleaved in a second site near the C-terminus, yielding two fragments of M_r 48K (Ts) and 4K (Ps-3). The small fragment (Ps-3) contains the C-terminus of the α-subunit and possibly the β-subunit. The data show that the M_r 20K fragment gradually decreased at advanced stages of digestion, yielding the M_r 4K fragment and also the M_r 10K fragment, both containing the C-terminus. The general process of tubulin-limited proteolysis can be summarized in the scheme of Figure 11. One of the most interesting functional consequences of limited proteolysis by subtilisin was the observation of the loss of MAP_2 in the assembled polymer, suggesting the absence of interactions or decreased interactions between higher molecular weight tubulin fragments (48K and 32K) in the polymer with MAP_2. Furthermore, the binding experiments of MAP_2 with digested fragments of tubulin indicated that the C-terminal fragments can bind to the high molecular weight protein MAP_2. The decreased incorporation of ^{125}I-MAP_2 in microtubules after pretreatment of the radiolabeled protein with increasing amounts of the C-terminal tubulin peptides as shown in Figure 10 also supports the involvement of the carboxyl-terminal region of tubulin in the interaction with MAP_2.

The carboxyl-terminal domain of tubulin has several characteristics. It has a similar sequence for both α- and β-tubulin subunits at least for the last 36 amino acids, and such a sequence is enriched in glutamic acid residues (40% of the residues in the last 36 amino acids) (Ponstingl et al., 1981; Krauhs et al., 1981). This domain is predicted to contain the main α-helix potential in both subunits (Ponstingl et al., 1983), a helix which can be stabilized by the addition of some cations if we consider the behavior of that domain similar to that of the polyglutamic acid (Jacobson, 1964). It is known that MAP_2 interacts with tubulin through a cationic domain present in a terminal end of the MAP_2 molecule (Vallee, 1980), and now we suggest that the carboxyl-terminal region of tubulin is involved in such an interaction.

Taking both results together, it seems reasonable to consider electrostatic interactions of a positively charged terminal domain of MAP_2 with the glutamic acid rich carboxyl-terminal region of tubulin subunits.

This information combined with future studies on chemical characteristics, conformational analysis of the C-terminal tubulin fragments, and biophysical studies on MAP_2 binding to tubulin would provide additional insight on the nature of tubulin–MAP_2 interactions.

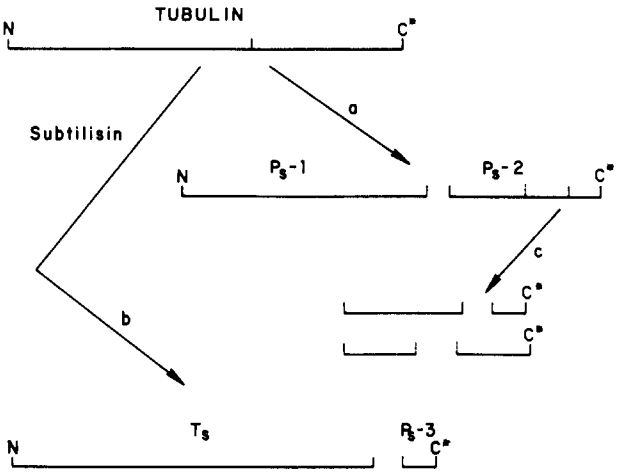

FIGURE 11: Schematic representation of tubulin's proteolysis by subtilisin. (a) Tubulin subunits treated with subtilisin at 30 °C generate the polypeptides Ps-1 and Ps-2 (M_r 32K and 20K, respectively). (b) When the digestion time interval increased (10 min), tubulin was nicked in a second cleavage point, generating Ts and Ps-3 (M_r 48K and 4K, respectively). (c) Ps-2 was cleaved into a smaller size fragment and Ps-3. The C-terminal residue (C*) is shown in tubulin and peptides Ps-2 and Ps-3. The dashed lines represent a partial digestion found sometimes for Ps-2.

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References

Anderson, B. L., Berry, R. W., & Telser, A. (1983) *Anal. Biochem. 132*, 365–373.

Appu-Rao, A. G., Hare, D. L., & Cann, J. R. (1978) *Biochemistry 17*, 4735–4739.

Arce, C. A., Rodriquez, J. A., Barra, H. S., & Caputto, R. (1975) *Eur. J. Biochem. 59*, 145–149.

Brown, H. R., & Erickson, H. P. (1983) *Arch. Biochem. Biophys. 220*, 46–51.

Carlier, M. F., Simon, C., & Pantaloni, D. (1980) *Biochem. Biophys. Res. Commun. 96*, 1761–1767.

Fairbanks, G., Steck, R. C., & Wallach, D. F. (1971) *Biochemistry 10*, 2606–2617.

Jacobson, A. L. (1964) *Biopolymers 2*, 237–244.

Johnson, K. A., & Borisy, G. G. (1975) in *Molecules and Cell Movement* (Inoue, S., & Stephens, R. E., Eds.) pp 119–141, Raven Press, New York.

Krauhs, E., Little, N., Kempf, T., Hofer-Warbineck, R., Ade, W., & Ponstingl, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4158–4160.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 9*, 265–275.

Ludueña, R. F., Roach, M. C., Brinkley, P., & Jordan, M. A. (1982) *J. Cell Biol. 95*, 347a.

Maccioni, R. B., & Seeds, N. W. (1981) *J. Cell Biol. 91*, 322a.

Maccioni, R. B., & Seeds, N. W. (1982) *Biochem. Biophys. Res. Commun. 108*, 896–903.

Maccioni, R. B., & Seeds, N. W. (1983) *Biochemistry 22*, 1567–1572.

Maccioni, R. B., Vera, J., & Slebe, J. (1981a) *Arch. Biochem. Biophys. 207*, 248–255.
Maccioni, R. B., Vera, J., & Slebe, J. (1981b) in *Molecular Approaches to Gene Expression and Protein Structure* (Siddiqui, M. A. Q., Krauskopf, M., & Weissbach, H., Eds.) pp 238–308, Academic Press, New York.
Mann, K., Giesel, M., Fasold, H., & Haase, W. (1978) *FEBS Lett. 92*, 45–48.
Mellado, R. P., Slebe, J., & Maccioni, R. B. (1980) *Biochem. Int. 1*, 584–590.
Mellado, W., Slebe, J., & Maccioni, R. B. (1982) *Biochem. J. 203*, 675–681.
Palanivelu, P., & Luduena, R. F. (1982) *J. Biol. Chem. 257*, 6311–6315.
Postingl, H., Krauhs, E., Little, M., & Kempf, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2757–2761.
Postingl, H., Krauhs, E., & Little, M. (1983) *J. Submicrosc. Cytol. 15*, 359–362.
Sandoval, I. V., & Weber, K. (1980) *J. Biol. Chem. 255*, 6966–6974.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.
Vallee, R. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3206–3210.
Vallee, R. B., & Borisy, G. (1977) *J. Biol. Chem. 252*, 377–382.
Vallee, R. B., & Borisy, G. (1978) *J. Biol. Chem. 253*, 2834–2845.
Wadsworth, P., & Sloboda, R. D. (1982) *Biochemistry 21*, 21–28.
Weingarten, M., Lockwood, A., Hwo, S., & Kirshner, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.

Comparison of Structures of Various Human Fibrinogens and a Derivative Thereof by a Study of the Kinetics of Release of Fibrinopeptides†

Lewis S. Hanna,‡ Harold A. Scheraga,* Charles W. Francis, and Victor J. Marder

ABSTRACT: The kinetics of the thrombin-induced release of fibrinopeptides from several variants of human fibrinogen, and from the plasmin digestion fragment E thereof, have been studied by using an HPLC technique to separate the reaction products. The data were analyzed in terms of a Michaelis–Menten mechanism in which the Aα and Bβ chains compete for thrombin. Phosphorylation of Ser-3 of the Aα chain appears to increase the rate of release of the corresponding phosphorylated peptide A from fibrinogen, due to enhanced binding of thrombin (lower value of the Michaelis–Menten constant K_M). However, phosphorylation does not affect the rate of release of the unphosphorylated A or B peptides. Increase in the length of the γ chain (at the C-terminus) does not affect the rate of release of any of the fibrinopeptides. The rate of release of the A peptide from fragment E (which is devoid of the B peptide) is similar to that for the complete fibrinogen molecule. These results are in agreement with an earlier conclusion [Martinelli, R. A., & Scheraga, H. A. (1980) *Biochemistry 19*, 2343] that the Aα and Bβ chains behave independently in their competition for thrombin; i.e., the hydrolyzable Arg–Gly bonds of the Aα and Bβ chains are both accessible to thrombin.

We are carrying out kinetic studies of the thrombin–fibrinogen reaction to determine the size of the active site of thrombin and the nature of its interactions with the fibrinogen substrate (Scheraga, 1977; Marsh et al., 1983). Various substrates are used in these investigations, viz., fibrinogen and several fragments thereof, and the efficiency with which thrombin hydrolyzes these substrates is expressed in terms of the ratio of the Michaelis–Menten parameters, k_{cat}/K_M. The values of k_{cat}/K_M for the hydrolysis of Arg–Gly bonds of the Aα and Bβ chains of fibrinogen itself, leading to the release of fibrinopeptides A and B (FpA and FpB),[1] respectively, serve as a reference against which to judge the suitability of various fibrinogen derivatives as substrates for thrombin. We have previously determined the values of k_{cat}/K_M for bovine fibrinogen (Martinelli & Scheraga, 1980), and we report here the corresponding values for human fibrinogen.

Besides comparing the kinetic data for the bovine and human species, we carried out this investigation primarily for three additional purposes, to determine (i) whether the reported effect of phosphorylation of human FpA on the clotting time (Blombäck et al., 1963) has its origin in the proteolytic action of thrombin on fibrinogen [which might imply an interaction of (phosphorylated) Ser-3 of FpA with thrombin or with other parts of the fibrinogen molecule to induce a more favorable conformation around the hydrolyzable Arg–Gly bond], (ii) whether the variable length at the C-termini of the γ chains of human fibrinogen (Mosesson et al., 1972; Francis et al.,

† From the Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853 (L.S.H. and H.A.S.), and the Hematology Unit, Department of Medicine, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642 (C.W.F. and V.J.M.). *Received February 28, 1984.* This work was supported by research grants from the National Heart, Lung and Blood Institute of the National Institutes of Health (Grants HL-30616, HL-01662, and HL-00733). This article is paper 14 in a series.

‡ National Institutes of Health Postdoctoral Fellow, 1981–1984.

[1] Abbreviations: F, fibrinogen; Aα and Bβ, the peptide chains of fibrinogen that release fibrinopeptides A and B, FpA and FpB, respectively, upon hydrolysis by thrombin; AP (or FpAP), a phosphorylated form of FpA; γ, the third type of chain (of variable length) in fibrinogen; D and E, C- and N-terminal fragments of F, produced by plasmin digestion; DSK, disulfide knot (Blombäck et al., 1972); CNBrAα and CNBrBβ, the Aα and Bβ chains, respectively, of the DSK; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; PEG 6000, polyethylene glycol (used to inhibit surface adsorption of thrombin); ε-ACA, ε-aminocaproic acid (a plasmin inhibitor); Trasylol, pancreatic trypsin inhibitor (used to inhibit trypsin, chymotrypsin, and other proteolytic enzymes); SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; ODS, octadecylsilane.

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