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Interaction of Microtubule-Associated Proteins with Microtubules: Yeast Lysyl- and Valyl-tRNA Synthetases and τ 218-235 Synthetic Peptide as Model Systems†

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ABSTRACT: The respective contributions of electrostatic interaction and specific sequence recognition in the binding of microtubule-associated proteins (MAPs) to microtubules have been studied, using as models yeast valyl- and lysyl-tRNA synthetases (VRS, KRS) that carry an exposed basic N-terminal domain, and a synthetic peptide reproducing the sequence 218-235 on τ protein, known to be part of the microtubule-binding site of MAPs. VRS and KRS bind to microtubules with a K_D in the 10^{-6} M range, and τ 218-235 binds with a K_D in the 10^{-4} M range. Binding of KRS and τ 218-235 is accompanied by stabilization and bundling of microtubules, without the intervention of an extraneous bundling protein. τ 218-235 binds to microtubules with a stoichiometry of 2 mol/mol of assembled tubulin dimer in agreement with the proposed binding sequences α[430-441] and β[422-434]. Binding stoichiometries of $2/\alpha\beta_S$ tubulin and $1/\alpha_S\beta_S$ tubulin were observed following partial or complete removal of the tubulin C-terminal regions by subtilisin, which localizes the site of subtilisin cleavage upstream residue α-441 and downstream residue β-434. Quantitative measurements show that binding of MAPs, KRS, VRS, and τ 218-235 is weakened but not abolished following subtilisin digestion of the C-terminus of tubulin, indicating that the binding site of MAPs is not restricted to the extreme C-terminus of tubulin.

Microtubules are dynamic cytoskeletal polymers involved in a variety of motile phenomena. Elucidating the mechanism of regulation of microtubule dynamics is a crucial issue in cell motility. The irreversible hydrolysis of tubulin-bound GTP that accompanies microtubule assembly is known to be at the origin of the dynamic instability behavior (Mitchison & Kirschner, 1984; Carlier, 1989). However, how dynamic instability is regulated in vivo is not understood yet. Accessory proteins (MAPs) that bind tightly to microtubules are known to stabilize the polymer. The most extensively studied of these MAPs are the brain proteins MAP-2 and τ (Olmsted, 1986;

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Wiche, 1989). A second class of MAPs are motor ATPases such as kinesin, dynein, dynamin, and related proteins that are responsible for directed transport of organelles and chromosome movement along microtubules (Vale, 1990; Shpetner & Vallee, 1989; Meluh & Rose, 1990). In addition, a large number of enzymes of the glycolytic pathway are known to bind to microtubules [for review see Walsh et al. (1989)]; these abundant proteins might affect microtubule dynamics in cells that do not contain MAP-2 or τ.

MAPs enhance microtubule assembly by binding to the highly acidic C-terminal extremities of α- and β-tubulins (Ponstingl et al., 1981; Krauhs et al., 1981; Littauer et al., 1986), which are exposed on the microtubule surface. Other conditions known to promote polymerization of tubulins are the removal of a 2–4-kDa fragment from their C-termini by controlled proteolysis with subtilisin (Serrano et al., 1984a,b; Maccioni et al., 1986; Sackett et al., 1985; De la Viña et al., 1988; Paschal et al., 1989) or the neutralization of the negative charges by polycations (Erickson & Voter, 1976; Lee et al., 1978; Kuznetsov et al., 1978), by Mg^{2+} and by selective modification of carboxyl groups (Mejillano & Himes, 1991). These findings have led to the long-held view that the driving force for enhanced polymerization of tubulin was the relief of charge repulsion between adjacent carboxyl termini in the polymer.

In recent years, impressive progress was made in delineating the binding sites on MAPs and tubulins that are involved in their association. MAP-2 (Lewis et al., 1988), τ (Lee et al., 1988; Aizawa et al., 1988), and MAP-U (Aizawa et al., 1990) and its human nonneuronal counterpart MAP-4 (Chapin & Bulinski, 1991) were shown to contain highly homologous octadecapeptide repeats located within their C-terminal basic region. Synthetic polypeptides reproducing such repeats from τ (Ennulat et al., 1989), MAP-2 (Joly et al., 1989; Joly & Purich, 1990), and MAP-U (Aizawa et al., 1989) bind to tubulin, promote microtubule assembly, and compete with MAPs for binding to tubulins. Moreover, proton nuclear magnetic resonance analysis of the binding to tubulin of a synthetic peptide corresponding to the first repeat from MAP-U has shown that hydrophobic as well as ionic interactions are involved (Kotani et al., 1990). Correlatively, compelling evidence was obtained that the sequences corresponding to residues 430–441 in α-tubulin and 422–434 in β-tubulin, which are conserved in their respective isotypes and are centrally located within the acidic C-terminal region, define, or at least are part of, the MAP-binding domains of tubulin. Synthetic polypeptides corresponding to these sequences inhibit MAP–induced polymerization of tubulin and bind to MAP-2 and τ (Maccioni et al., 1988), as well as to the first and second repeats of τ, inducing conformational changes (Maccioni et al., 1989). Moreover, antibodies to these polypeptides inhibit MAP-promoted polymerization (Vera et al., 1988) while anti-idiotypic antibodies to these antibodies react with MAP-2 or τ (Rivas et al., 1988; Rivas-Berios et al., 1990). It should be noted that the concentration of these MAP- or tubulin-derived synthetic peptides required to elicit the above-mentioned effects is at least 2 orders of magnitude higher than that needed using the corresponding intact molecules. This clearly indicates that the regions flanking these sequences in the intact molecules, which are rich in cationic residues in the case of MAPs and highly anionic in the case of tubulin, contribute to MAP–tubulin association, through ionic interactions. It has been proposed that this association involves not only electrostatic charge neutralization but also the above-mentioned sequence-specific interactions that induce conformational changes required for efficient assembly into microtubules (Kotani et al., 1990).

To further explore the contribution of charge neutralization to the enhancement of tubulin polymerization, we have used as models two eukaryotic aminoacyl-tRNA synthetases that carry an exposed polycationic N-terminal domain of limited size and known sequence. Compared to their prokaryotic homologues, yeast valyl- and lysyl-tRNA synthetases display N-terminal extensions that are rich in lysine residues (Chatton et al., 1988; Mirande & Waller, 1988). A portion of these extensions, corresponding to residues 32–54 (**KEIEK-EKKKAEKLLKFAAKQAKK**) in valyl-tRNA synthetase and 33–53 (**KKRIKQRQVEAKKAAKKAKKAAAQ**) in lysyl-tRNA synthetase, have a potential to form an amphiphilic α-helix in which, respectively, 10 and 7 lysine residues are aligned on one face of the helix. We have studied the binding of these enzymes to normal and subtilisin-treated microtubules and their effect on tubulin polymerization, in the absence or presence of MAPs or of a synthetic peptide reproducing the second octadecapeptide repeat from τ, corresponding to residues 218–235 (VT**SK**CGSLGN**IHHK**PGGG).

The results provide a new insight into the mechanism of microtubule bundling and in the location of MAP-binding sites of tubulin.

Materials and Methods

Chemicals. 2-(*N*-Morpholino)ethanesulfonic acid (MES) was purchased from Calbiochem. Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), dithioerythritol (DTE), sodium dodecyl sulfate (SDS), papain, and subtilisin Carlsberg (P 5380) were from Sigma. Guanosine 5′-triphosphate (GTP) and yeast total tRNA were from Boehringer. Acrylamide and all other gel electrophoresis reagents came from Bio-Rad. Taxol was a gift of D. Guénard (ICSN, Gif-sur-Yvette, France). [^{14}C]-L-Lysine and [^{14}C]-L-valine came from CEA—Saclay (France) and [^{14}C]iodoacetamide from NEN. All other chemicals were analytical grade from Merck.

Purification of Tubulin and Microtubule-Associated Proteins (MAPs). Whole microtubule protein was prepared from fresh pig brain by three cycles of assembly according to Shelanski et al. (1973). Pure tubulin was further isolated from microtubule proteins by phosphocellulose (Whatman P11) chromatography (Weingarten et al., 1975), concentrated by ultrafiltration, and stored at −80 °C in MG buffer (0.05M MES, pH 6.8, 0.5 mM EGTA, 0.25 mM $MgCl_2$, 3.4 M glycerol, and 200 μM GTP) at a concentration of 5–10 mg/mL.

MAPs were isolated from whole microtubule protein by phosphocellulose and DEAE-Sephadex chromatography according to Kuznetsov et al. (1981) and stored at −80 °C in MG buffer.

Purification of Aminoacyl-tRNA Synthetases. Cytoplasmic lysyl- and valyl-tRNA synthetases (KRS and VRS, respectively) were purified to homogeneity from exponentially grown *Saccharomyces cerevisiae* strain D273 (Cirakoglu & Waller, 1985; Bec & Waller, 1989). The stock solutions in 50 mM potassium phosphate, pH 7.0, 1 mM dithioerythritol, and 50% glycerol were stored at −20 °C. KRS is composed of two identical subunits of 67 881 Da, while VRS is a monomer of 120 128 Da.

Proteolytic Digestions. Cleavage of the C-terminal region of tubulin by subtilisin (Carlsberg) was performed under different conditions to obtain either homogeneous $\alpha\beta_S$ tubulin in which only the β subunit was cleaved or homogeneous $\alpha_S\beta_S$ tubulin which had both subunits cleaved. Bhattacharyya et al. (1985) first reported that the β subunit was cleaved at a

3–4-fold faster rate than the α subunit on dimeric tubulin. We found that this difference in the sensitivity of α and β to subtilisin attack is greatly enhanced when tubulin is assembled in microtubules. SDS–PAGE monitoring of the cleavage reaction showed that, upon treatment of Taxol-stabilized microtubules (20 μM tubulin polymerized in MG buffer containing 1 mM GTP) by subtilisin at 20 μg/mL, removal of the C-terminus of β-tubulin was complete within 4–6 min at 37 °C, while the α subunit remained intact for at least 2 h. Therefore, microtubules entirely made of $\alpha\beta_S$ tubulin were obtained by a 15-min treatment of Taxol-stabilized microtubules by subtilisin under the conditions described above. On the other hand, overnight treatment of Taxol-stabilized microtubules by subtilisin as described by Rodionov et al. (1990) yielded S-microtubules entirely made of $\alpha_S\beta_S$ tubulin. Subtilisin-treated microtubules were then sedimented through a cushion of 30% sucrose in MG buffer supplemented with 5 μM Taxol and 2 mM PMSF at 170000*g*, 25 °C, for 1 h. Pellets were washed and resuspended in MG buffer containing 20 μM Taxol and 2 mM PMSF and stored at −80 °C at a concentration of 1 mg/mL.

Alternatively, dimeric tubulin was digested by subtilisin using the conditions described by Sackett et al. (1985) to obtain $\alpha_S\beta_S$ tubulin. The $\alpha_S\beta_S$ products obtained by the two latter methods displayed identical gel electrophoresis patterns and also behaved identically regarding MAPs and KRS or VRS binding (see Results).

The lysine-rich N-terminal domain of yeast lysyl-tRNA synthetase was selectively cleaved by papain digestion as previously described, yielding an active enzyme that no longer binds to polyanionic carriers (Cirakoglu & Waller, 1985).

Electron Microscopy. Samples of tubulin polymerized at 12–15 μM were negatively stained (without fixation) on carbon-coated grids with 1% uranyl acetate and viewed in a Philips EM410 electron microscope.

Polymerization Measurements. Tubulin assembly into microtubules was monitored by turbidimetry at 350 nm in a spectrophotometer equipped with a 120-μL cuvette thermostated at 37 °C (0.5-cm light path). Polymerization buffer for pure tubulin assembly was MG buffer supplemented with 6 mM $MgCl_2$, except when polymerization was assayed in the presence of peptide τ-2, in which case MG buffer containing only 0.5 mM $MgCl_2$ was used. Whole microtubule protein was assembled in MM buffer (0.1 M MES, pH 6.8, 1 mM EGTA, 0.5 mM $MgCl_2$).

Sedimentation Assay for Binding of Lysyl- and Valyl-tRNA Synthetases to Microtubules. Binding of VRS or KRS to microtubules was determined using the following standard sedimentation assay. Tubulin (15–20 μM) in MG buffer at 0 °C containing 6 mM $MgCl_2$ and 0.4 mM GTP was split into a series of 150-μL Airfuge tubes containing different amounts of VRS or KRS. The samples were then incubated at 37 °C for 30 min and spun at 170000*g* for 4 min in the Airfuge (Beckman) thermostated at 30 °C by a flow of warm air. The amount of VRS and KRS bound to microtubules was derived from the measurements of aminoacyl-tRNA synthetase activity in the samples before centrifugation and in the supernatants of sedimented microtubules, using the tRNA aminoacylation assay described by Bec et al. (1989). Protein concentration in the supernatants was assayed by the Lowry (1951) or the Bradford (1976) method. The difference between the concentration of total protein and that of VRS or KRS in the supernatants represents the concentration of nonassembled tubulin. The amounts of VRS, KRS, and tubulin present in the pellets and supernatants could also be estimated by SDS–PAGE of all fractions following denaturation. The same sedimentation assay combined with SDS gel electrophoresis was used to measure MAP binding to microtubules.

SDS–Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed in 1.5 mm thick slab gels composed of 8% acrylamide and 0.2% methylenebis(acrylamide) and using the following modification of the method of Laemmli (1970) in order to increase the separation of the α and β subunits of tubulin. The pH of the running gel was 9.5 and the electrode buffer contained 0.1% SDS coming from Sigma. Samples were denatured by boiling for 3 min in the presence of 2% SDS, 5% β-mercaptoethanol, 30% glycerol, and 0.001% bromophenol blue in 0.06 M Tris-HCl, pH 6.8. Electrophoresis was carried out for 15 h under 35 V. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 5% acetic acid and 40% ethanol in H_2O.

Peptide Synthesis and Labeling. The octadecapeptide corresponding to the second of the three imperfect repeats of τ protein (τ[218–235]) (Ennulat et al., 1989) was kindly synthesized by Pr. P. Cohen and Dr. H. Boussetta using a Neosystem NPS 4000 instrument (Strasbourg, France) as described by Nicolas et al. (1986). The peptide (τ-2) had the following sequence: VTSKCGSLGNIHHKPGGG. The synthesized product was purified by HPLC on a Partisil ODS3 column with a linear gradient of 0–60% acetonitrile in 0.5% trifluoroacetic acid. The purified peptide was stored desiccated at −20 °C after lyophilization. The amino acid composition of the purified peptide was analyzed after total hydrolysis in 6 N HCl for 24 h in order to check the purity and to determine the concentration of the peptide.

Radioactive labeling of the peptide was achieved by carboxymethylation of its single cysteine by [^{14}C]iodoacetamide. The peptide, at a concentration of 5 mM, was reacted with 7.5 mM [^{14}C]iodoacetamide (2.67 Ci/mol) in 25 mM MES, pH 6.8, at 4 °C overnight. The reaction was stopped by addition of 75 mM β-mercaptoethanol, and the material was extensively dialyzed against 2 L of 25 mM MES, pH 6.8 (two changes), using Spectra/Por-1000 dialysis membranes (Spectrum) and stored at −20 °C. It was verified that the labeled peptide promoted microtubule assembly with exactly the same efficiency, at different concentrations, as the unlabeled peptide.

Results

Microtubule Assembly in the Presence of Lysyl- or Valyl-tRNA Synthetases. Tubulin (15 μM) was polymerized, in MG buffer supplemented with 6 mM Mg^{2+}, in the presence of different amounts (0–20 μM) of either KRS or VRS. Turbidimetric recordings of the polymerization process are shown in Figure 1A,B. In the presence of increasing amounts of KRS in the range 0–5 μM (Figure 1A), the lag time preceding the onset of assembly progressively decreased, while the extent of turbidity change increased up to 4–5-fold, i.e., much more than the 50–75% increase expected if the critical concentration decreased to zero. Upon increasing the KRS concentration above 5 μM, the maximum extent of turbidity change decreased back to zero, showing inhibition of polymerization at high concentration of KRS. When KRS was replaced by papain-digested KRS, from which the N-terminal domain was excised, none of the above effects was observed and tubulin polymerization was unaffected. This clearly indicates that the interaction of KRS with microtubules involves the positive charges clustered in its N-terminal domain.

The effects of VRS on tubulin polymerization were strikingly different: in the presence of increasing amounts of VRS in the range 0–2 μM, no detectable change in lag time was

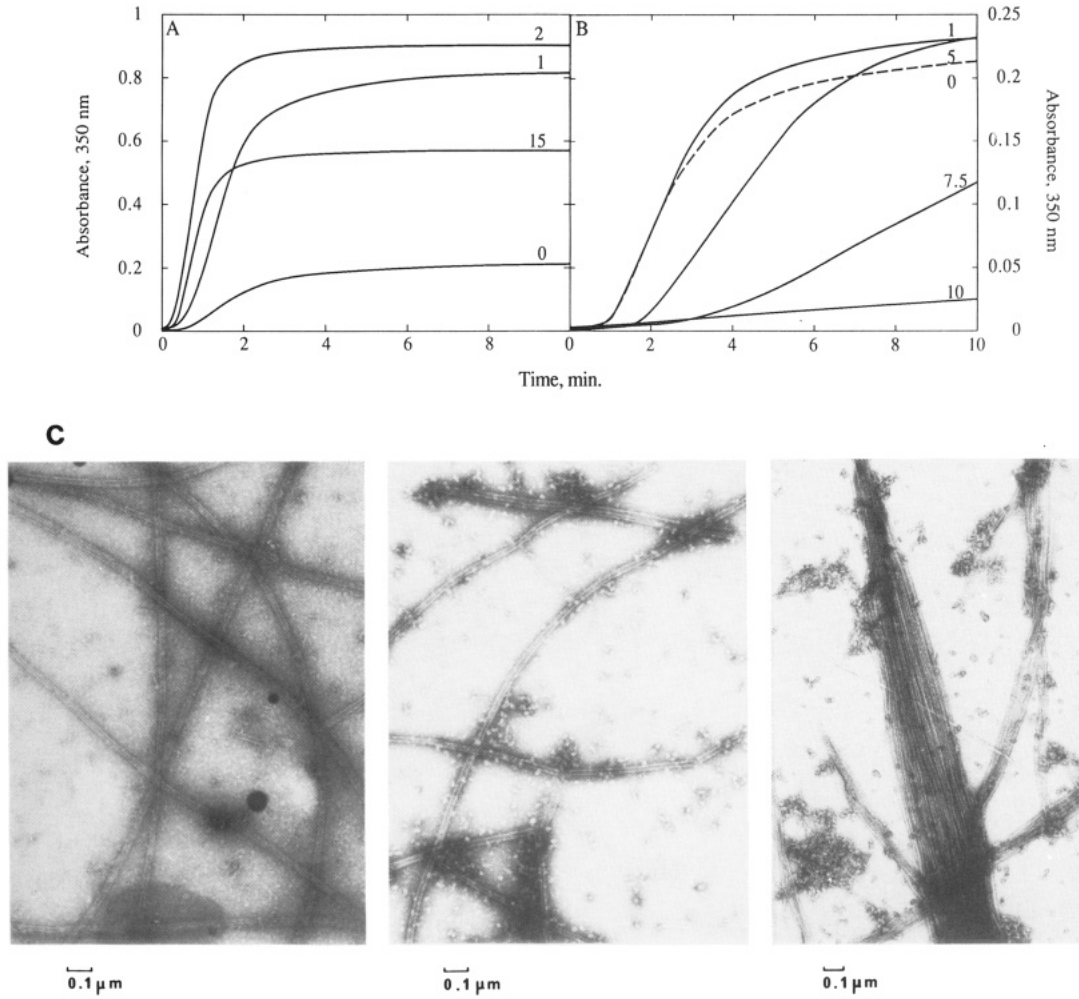

FIGURE 1: Tubulin assembly in the presence of KRS and VRS. Tubulin was polymerized at 15 μM in MG buffer in the presence of KRS (panel A) and VRS (panel B) at the indicated concentrations (in μM). Turbidity at 350 nm was recorded (light path 0.5 cm). Panel C shows electron micrographs of species assembled in the absence of aminoacyl-tRNA synthetases (left panel) and in the presence of 1 μM VRS (center panel, single microtubules) and of 1 μM KRS (right panel, microtubule bundles).

observed and the extent of turbidity change increased only slightly (up to about 10%); above 2 μM VRS, tubulin polymerization was increasingly inhibited, as observed above with KRS. With both aminoacyl-tRNA synthetases, it was verified that the same turbidity level was reached when the synthetases were added to preassembled microtubules or to tubulin prior to polymerization. In addition, microtubules assembled in the presence of VRS or KRS completely disassemble upon cooling the solution to 4 °C.

Electron microscopy of negatively stained samples of tubulin polymerized in the presence of 1 μM KRS showed microtubules packed in bundles, while single normal microtubules were observed in preparations of tubulin polymerized in the presence of 1 μM VRS. KRS-induced aggregation of microtubules accounts for the very large increase in turbidity displayed in Figure 1A.

As will be discussed later, the differences in the behavior of VRS and KRS may be related to their monomeric and dimeric structures, respectively.

Quantitative Analysis of Lysyl- or Valyl-tRNA Synthetase Binding to Microtubules. The binding of KRS and VRS to microtubules was monitored using the sedimentation assay described under Materials and Methods. The amount of KRS bound to microtubules and the amount of tubulin polymerized are plotted as a function of KRS concentration in Figure 2A, and SDS–PAGE of the pellets and supernatants of representative samples are shown in Figure 2B. Consistent with the preceding turbidity observations, the data demonstrate that in a range of low concentrations (0–5 μM) of KRS, the enzyme binds to microtubules and accordingly causes a decrease in tubulin critical concentration, while at higher KRS concentration (>7 μM), binding of KRS to tubulin prevents its polymerization; i.e., KRS sequesters tubulin and both proteins are found in the supernatant of sedimented microtubules. Analysis of KRS binding to microtubules (Figure 2A, inset) shows that a maximum of about 0.8 KRS was bound per polymerized tubulin. The equilibrium dissociation constant for KRS binding to polymerized tubulin could also be derived from the analysis of binding data in the range 0.05–1 μM KRS, assuming that KRS binds to microtubules exclusively (and not to dimeric tubulin) in this low concentration range. The double-reciprocal plot of the data shown in Figure 2A led to a value of 2 μM for the equilibrium dissociation constant (K_D) for KRS binding to microtubules and a binding stoichiometry of 1 KRS per polymerized tubulin $\alpha\beta$ dimer.

The binding of VRS to microtubules and tubulin was analyzed as described for KRS. Qualitatively similar conclusions were derived, namely, VRS binds to polymerized tubulin at low concentrations, while at higher concentrations, it prevents polymerization by sequestring tubulin. The data, displayed in Figure 3, show that VRS binds to microtubules with a lower affinity ($K_D \sim 5$–10 μM) than KRS. Although the extrapolated stoichiometry, on the calculated isotherm, is one VRS bound per polymerized tubulin, the extent of VRS binding to

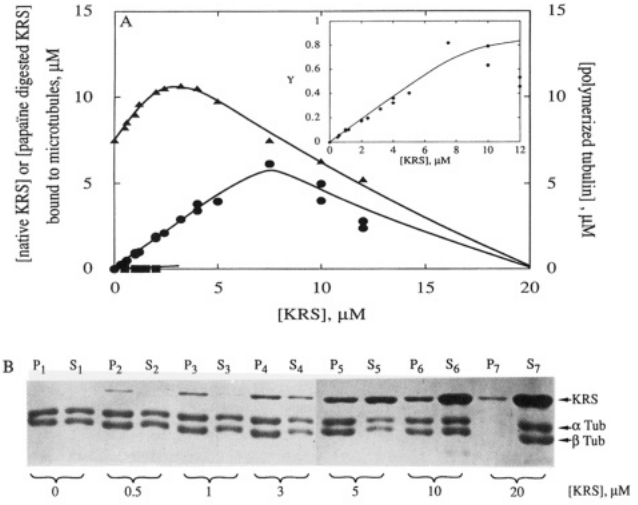

FIGURE 2: Binding of KRS to microtubules. Panel A: Relationship between bound KRS and polymerized tubulin. Tubulin (15 μM) in MG buffer was polymerized for 30 min in the presence of KRS at the indicated concentrations. The sedimentation assay described under Materials and Methods was used to quantitate the amounts of microtubule-bound KRS (●) and polymerized tubulin (▲). A control was done using papain-cleaved KRS (■) that does not bind to microtubules. The inset shows the molar ratio of KRS per tubulin in microtubules, Y, as derived from data in the main graph. The curve (solid line) represents the isotherm calculated for a binding ratio of 1 mol of KRS/mol of assembled tubulin and an equilibrium dissociation constant of 2 μM. Panel B: SDS–PAGE patterns of pellet (P_i) and supernatant (S_i) fractions of tubulin polymerized in the presence of the indicated concentrations of KRS.

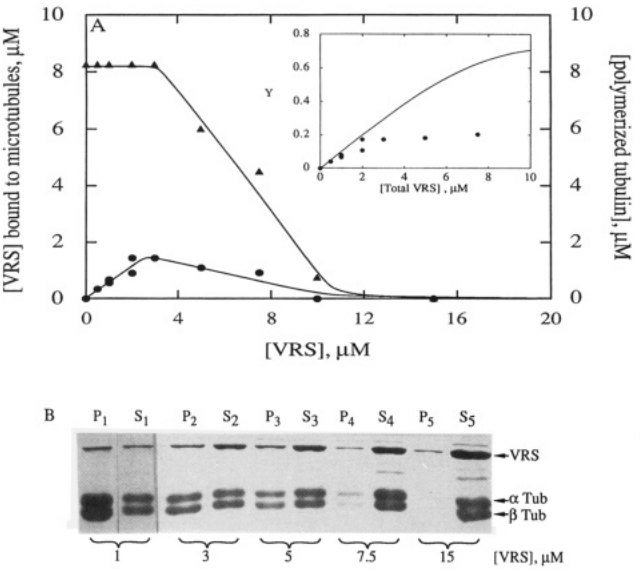

FIGURE 3: Binding of VRS to microtubules. The experiment was conducted and the data are represented as described in the legend to Figure 2, with VRS in the place of KRS.

microtubules, in the experiment, never exceeded 0.2 VRS bound per assembled tubulin. The fact that KRS is a dimer most likely accounts for its higher affinity, first, because it possesses a twice larger positive charge per mole, and second, two clusters of positive charges are localized within a short distance on the same molecule, which may also contribute to increase the affinity for the polymer by binding to two adjacent tubulins.

Competition between Valyl- and Lysyl-tRNA Synthetases for Binding to Microtubules. The experiments described above and the similarity of the N-terminal polycationic domains of VRS and KRS suggested that their binding site on tubulin may overlap. This hypothesis was tested in competition ex-

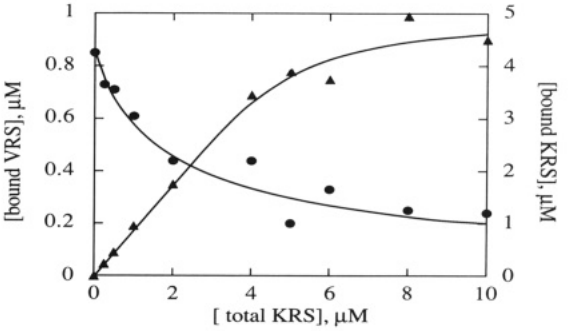

FIGURE 4: Competition between KRS and VRS for binding to microtubules. Tubulin (15 μM) was polymerized for 20 min in MG buffer in the presence of 1 μM VRS, 20 μM Taxol, and increasing amounts of KRS. The amounts of KRS (▲) and VRS (●) bound to sedimented microtubules were derived from measurements of the catalytic activity of the two enzymes in the sample before sedimentation and in the supernatants of sedimented samples.

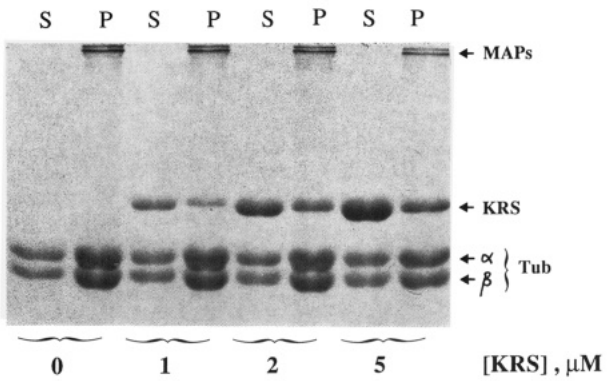

FIGURE 5: Binding of KRS to MAP-containing microtubules. Three times cycled whole microtubule protein (17.5 μM tubulin) was polymerized in 0.1 M MES, pH 6.8, 1 mM EGTA, and 0.5 mM $MgCl_2$ in the presence of KRS at the indicated concentrations. SDS–PAGE profiles of the pellets (P_i) and supernatants (S_i) of sedimented samples are shown.

periments as follows. Tubulin (15 μM) was polymerized in the presence of 20 μM Taxol, 1 μM VRS, and increasing amounts of KRS in the range 0–10 μM. The amounts of VRS and KRS bound to microtubules were derived from measurements of the activity of the aminoacyl-tRNA synthetases in the samples before centrifugation and in the supernatants of sedimented microtubules. Figure 4 shows that VRS initially bound to microtubules is displaced by KRS, both proteins competing for the same site(s) on polymerized tubulin.

Competition between MAPs, τ-2 Peptide, and Lysyl- or Valyl-tRNA Synthetases for Binding to Microtubules. MAPs are known to bind to the acidic C-terminal region of tubulin that is exposed on microtubules. The possible competition between MAPs and KRS was investigated using the sedimentation assay described under Materials and Methods. MAP-containing microtubules were assembled in the presence of increasing concentrations of KRS (0–5 μM) in MM buffer. Figure 5 shows that about 25% of the KRS was bound to MAP-containing microtubules in this concentration range, while 80% of KRS had been found bound to microtubules assembled from pure tubulin (Figure 1). On the other hand, MAPs were not displaced from microtubules by KRS up to 5 μM. Since MAPs bind tightly to a lattice of sites on microtubules, with a maximum stoichiometry of 1 MAP/6–10 tubulin dimers, the present results suggest that KRS binds to "vacant" tubulin subunits in MAP-containing microtubules, with an affinity that is lowered by steric hindrance caused by MAPs. Due to the combined low binding stoichiometry and high affinity of MAPs for microtubules, extremely high con-

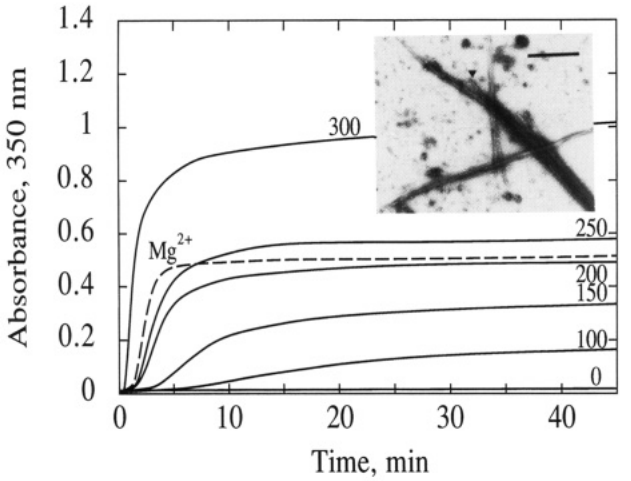

FIGURE 6: Stimulation of tubulin assembly by τ-2 synthetic peptide. Pure tubulin (15 μM) in 0.05 M MES, pH 6.8, 30% glycerol, 0.5 mM EGTA, and 0.25 mM $MgCl_2$ was polymerized in the presence of τ-2 synthetic peptide at the indicated concentrations (in μM). As a reference, the time course of tubulin polymerization upon addition of 6 mM $MgCl_2$ is shown (dashed line). Turbidity at 350 nm was recorded. Inset: Electron micrograph of microtubule bundles induced by τ-2 synthetic peptide (300 μM). Arrow indicates a microtubule fraying off a bundle. Bar = 1 μm.

centrations of KRS would be needed to displace MAPs.

That KRS and VRS nevertheless bind to the same region as MAPs was assessed using the τ 218–235 (τ-2) synthetic peptide representing the second of the three 18 amino acid imperfect repeats that have been shown to bind to microtubules (Ennulat et al., 1989; Maccioni et al., 1989). In a first experiment, the MAP-like activation of tubulin polymerization by τ-2 synthetic peptide was verified, by polymerizing pure tubulin (15 μM, in MG buffer) in the presence of different amounts of τ-2 peptide. Polymerization was monitored turbidimetrically. Figure 6 shows that, in agreement with a previous report (Ennulat et al., 1989), τ-2 peptide promoted tubulin polymerization in a concentration-dependent fashion. Microtubules assembled in the presence of τ-2 disassemble upon cooling the solution. Normal single microtubules were observed in the EM in the presence of up to 200 μM τ-2. However, at higher peptide concentrations, the extent of turbidity change increased dramatically, consistent with the observation of microtubule bundles in the electron microscope (inset). Pure tubulin (15 μM) was next polymerized in the presence of 1 μM either KRS or VRS, and of increasing amounts of τ-2 peptide. KRS and VRS were displaced from microtubules by the peptide, which therefore binds to the same region on tubulin. The concentration of τ-2 peptide necessary to obtain half-inhibition of the binding of 0.5 and 1 μM KRS or VRS was 0.6 and 1 mM, respectively, suggesting that τ-2 peptide binds to polymerized tubulin with an equilibrium dissociation constant in the 10^{-4} M range, in agreement with the value previously reported (Maccioni et al., 1989) for the same peptide and those reported for a related peptide derived from MAP-2 (Aizawa et al., 1989; Joly & Purich, 1990).

On the other hand, MAPs could not be displaced from whole microtubule proteins by τ-2 peptide added at concentrations up to 3 mM. This result is understandable given (i) the 10^3-fold higher affinity of MAPs for microtubules as compared to τ-2 peptide and (ii) the fact that MAPs bind substoichiometrically to polymerized tubulin, so that displacement of bound MAPs would necessitate a very high binding ratio to τ-2.

Size of the C-Terminal Region of Tubulin Responsible for the Binding of MAPs, τ-2 Peptide, and Lysyl- or Valyl-tRNA Synthetases. Although it is well established that MAPs interact, via electrostatic bonds, with the acidic C-terminal regions of α- and β-tubulin, agreement has not been reached about the size of the binding region. The origin of these discrepancies lies in conflicting reports concerning the site of cleavage of the C-terminus of tubulin by subtilisin. Some observations (Maccioni et al., 1986; Vera et al., 1989) led the authors to conclude that subtilisin cleavage occurs at residues 418 and 408 on α- and β-tubulin, respectively, i.e., that over 30 C-terminal amino acids are removed by subtilisin, while other data (Sackett et al., 1985; De la Viña et al., 1988; Paschal et al., 1989) rather indicate that only 5–13 amino acids are cleaved off tubulin C-terminus by subtilisin. In the following experiments, the subtilisin cleavage sites were defined with respect to the binding sites of τ-2 peptide on tubulins α and β.

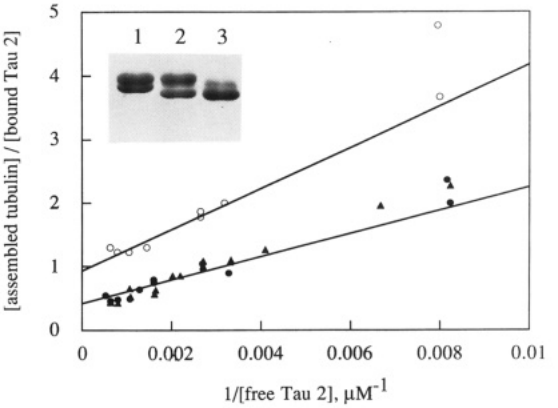

FIGURE 7: Double-reciprocal plots of the binding of [^{14}C]carboxymethylated τ-2 peptide to microtubules assembled from unmodified or subtilisin-cleaved tubulin. Increasing concentrations of radiolabeled τ-2 peptide were added to microtubules assembled from unmodified tubulin at 13 μM (●) or from partially subtilisin-digested $\alpha\beta_S$-tubulin (▲) or from $\alpha_S\beta_S$-tubulin (○) at 11 μM. Polymerization buffer was MG buffer containing 200 μM GTP, 20 μM Taxol, and either 3 mM $MgCl_2$ (for polymerization of unmodified tubulin) or 1 mM $MgCl_2$ (for polymerization of subtilisin-treated tubulin). The amounts of bound and free peptide were determined using the sedimentation assay described under Materials and Methods. In the calculation of the amount of bound τ-2, the amount of labeled peptide trapped in the pellet interstitial volume was taken into account using a control sample containing radioactive glucose. Inset: SDS gel electrophoresis profile of intact (1) and subtilisin-cleaved microtubules: $\alpha\beta_S$ (2), $\alpha_S\beta_S$ (3).

Binding of τ-2 peptide to microtubules made of pure unmodified tubulin and subtilisin-treated $\alpha\beta_S$ and $\alpha_S\beta_S$ tubulins was examined in a sedimentation assay using [^{14}C]τ-2, prepared as described under Materials and Methods. The three forms of tubulin were polymerized in the presence of 1.5 molar equivalent Taxol and labeled peptide at a series of concentrations. The presence of Taxol ensured 100% polymerization of tubulin, which was verified by protein measurements in the supernatants of sedimented samples. The binding data presented in Figure 7 show that τ-2 bound to microtubules assembled from either unmodified tubulin or partially cleaved $\alpha\beta_S$ tubulin with a stoichiometry of 2.4 mol of peptide/mol of tubulin dimer and an equilibrium dissociation constant of 0.4 mM. In contrast, τ-2 bound to microtubules assembled from $\alpha_S\beta_S$ tubulin with a lower stoichiometry (1.1 mol/mol of $\alpha_S\beta_S$ dimer) and the same equilibrium dissociation constant. These results are consistent with one peptide binding site on each subunit of tubulin and further demonstrate that subtilisin cleavage removes the peptide-binding site from the α subunit, but not from the β subunit. This last piece of data leads us to propose that the site of subtilisin cleavage would be around residue 430 on both subunits, which supports the data of

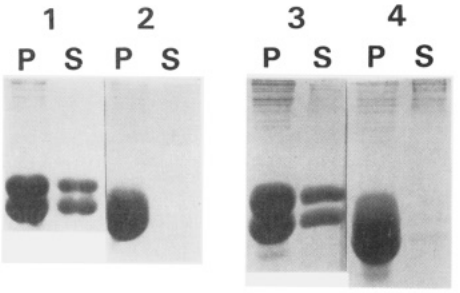

FIGURE 8: Decrease in affinity of MAPs for microtubules following cleavage of tubulin by subtilisin. DEAE-Sephadex-purified MAPs were added at a concentration of 0.075 mg/mL to Taxol-stabilized microtubules assembled from either unmodified or subtilisin-cleaved tubulin (15 μM) in MG buffer containing 3 mM $MgCl_2$, 200 μM GTP, and 15 μM Taxol. S-Microtubules in this experiment were obtained as described by Rodionov et al. (1990); however, identical results were obtained using S-tubulin prepared according to Sackett et al. (1985). Samples (100 μL) were either subjected to the standard sedimentation assay described under Materials and Methods (lanes 1 and 2) or deposited on a 200-μL cushion of 30% sucrose in the same buffer (lanes 3 and 4) and sedimented at 37 °C, 250000g, for 9 min in the TLA 100.1 rotor of the TL100 ultracentrifuge (Beckman). Pellet (P_i) and supernatant (S_i) fractions were processed for SDS–PAGE. The electrophoresis patterns of samples containing unmodified tubulin are shown in lanes 1 and 3, those containing S-tubulin, in lanes 2 and 4.

Sackett et al. (1985) and De la Viña et al. (1988) but disagrees with those of Maccioni's group (Serrano et al., 1984a,b; Maccioni et al., 1986; Vera et al., 1989). Within this view, subtilisin cleavage would remove the conserved sequence α-[430–438] but not the conserved sequence β[422–430] that have been proposed to be part of the binding site of τ (Maccioni et al., 1989).

The binding of MAPs and KRS or VRS to microtubules assembled from unmodified and $\alpha_S\beta_S$-tubulin was investigated comparatively. Using the standard sedimentation assay described under Materials and Methods, MAPs were found to cosediment identically with both types of microtubules (Figure 8, lanes 1 and 2). However, when the samples were centrifuged over a sucrose cushion, as designed by Rodionov et al. (1990), MAPs were found totally bound to normal microtubules, while over 50% of the MAPs were recovered in the supernatants of microtubules assembled from $\alpha_S\beta_S$-tubulin (Figure 8, lanes 3 and 4). It should be noted that the proportion of MAPs in the supernatants of S-microtubules, but not of normal microtubules, increases with the size of the sucrose cushion. These results indicate that MAPs can still bind to $\alpha_S\beta_S$-tubulin, albeit with a reduced affinity. Examination of the SDS gel electrophoresis pattern of $\alpha_S\beta_S$-tubulin as compared to that of unmodified tubulin shows that subtilisin cleavage of the C-termini went to completion. In addition, identical results concerning the binding of MAPs and KRS were obtained using $\alpha_S\beta_S$-tubulin prepared as described by Rodionov et al. (1990) or by Sackett et al. (1985) and Bhattacharyya et al. (1985). These two materials also exhibited identical patterns in SDS gel electrophoresis.

In order to further document the quantitative difference in the electrostatic interaction of MAPs and KRS with unmodified and subtilisin-treated ($\alpha_S\beta_S$) tubulin, binding of these proteins to the two types of microtubules was assayed in the presence of increasing concentrations of KCl. Microtubules were polymerized in the presence of Taxol, to avoid any depolymerization upon increasing ionic strength. The only effect of KCl was to decrease the strength of the interactions between MAPs or KRS and microtubules. SDS–PAGE of the pellets and supernatants allowed the determination of the KCl concentration at which 95% of these proteins were detached from microtubules. The data presented in Figure 9 show that the interaction of KRS and MAPs with microtubules was much more sensitive to ionic strength when microtubules were assembled from $\alpha_S\beta_S$-tubulin. This last result leads to the conclusion that the interaction of MAPs and KRS with microtubules is weakened by removal of the C-terminal region of α and β subunits by subtilisin, but a large portion of the electrostatic component of the interaction remains present. This conclusion is also in agreement with the location of the sites of subtilisin cleavage within the conserved sequence

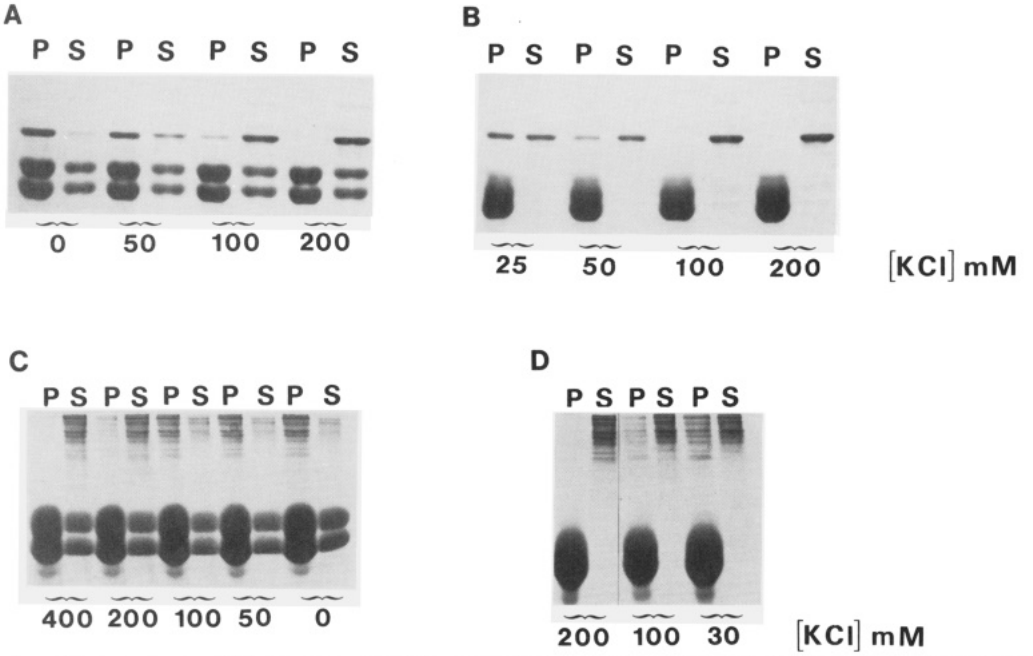

FIGURE 9: Ionic strength dependence of the binding of MAPs and KRS to microtubules assembled from unmodified and subtilisin-cleaved tubulin. KCl, at the indicated concentrations (in mM), was added to microtubules assembled from unmodified (A, C) or subtilisin-cleaved (B, D) tubulin (15 μM) in MG buffer containing 200 μM GTP and 20 μM Taxol, in the presence of KRS (1 μM, panels A and B) or high molecular weight MAPs (0.35 mg/mL, panels C and D). SDS gel electrophoresis profiles of the pellet and supernatant fractions of all samples are shown.

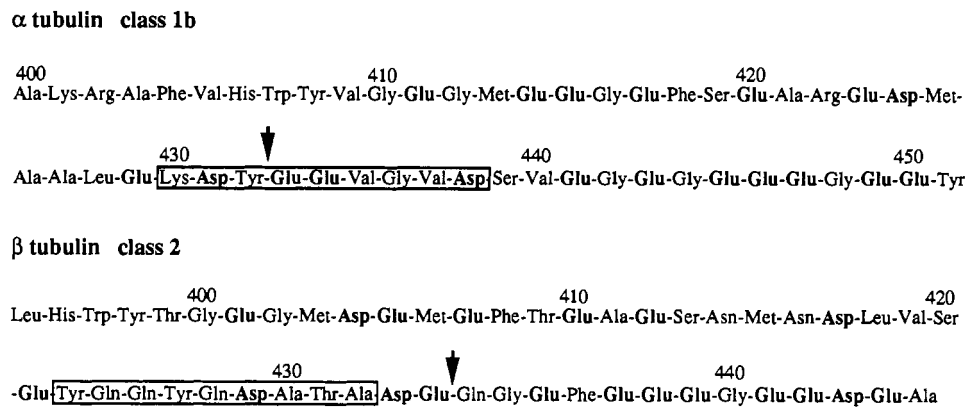

FIGURE 10: Carboxyl-terminal sequences of the α and β subunits of the major isotypes of pig brain tubulin (Little & Seehaus, 1988). Boxes indicate the sequences conserved in α and β isotypes, which represent the binding sites of the 18 amino acid repeats from τ and MAP-2, as determined by Maccioni et al. (1989). According to the results from the present study, substilisin cleavage abolishes the τ-peptide-binding site on the α subunits but not on the β subunits, in agreement with the subtilisin cleavage sites (arrows) proposed by De la Viña et al. (1988).

(residues 430–441) of the α subunits, and on the C-terminal side of conserved sequence (residues 422–434) of the β subunits, as proposed by De la Viña et al. (1989). According to this scheme, nearly half of the C-terminal anionic residues remain present following subtilisin cleavage (Figure 10).

Discussion

The high-affinity binding of MAPs to microtubules and the resulting stabilization of the polymer appear to be due to the combination of electrostatic interactions and specific sequence recognition. These two components of the interaction have been studied separately in the present work, using as models two aminoacyl-tRNA synthetases (valyl- and lysyl-tRNA synthetases) that carry an exposed polycationic N-terminal domain of limited size and a synthetic peptide reproducing the sequence 218–235 on τ protein, which is one of the three imperfect 18 amino acid repeats known to bind to microtubules.

Limited proteolysis of KRS has shown that the lysine-rich region of this protein was the binding site to microtubules, suggesting that the interaction was mainly electrostatic and occurred in a MAP-like fashion with the acidic C-terminal region of α- and β-tubulin. KRS and VRS bind to assembled tubulin with equilibrium dissociation constants of 1–10 μM, which is the range of average concentration of these enzymes in the cell. The physiological relevance, if any, of these interactions observed in vitro remains to be established. It was previously proposed that the polycationic extensions that characterize most of the lower eukaryotic aminoacyl-tRNA synthetases may ensure their spatial confinement at or close to the sites of protein biosynthesis [Cirakoglu & Waller, 1985; reviewed in Mirande (1991)].

The binding of KRS to microtubules is linked to stabilization of the polymer, as indicated by a decrease in critical concentration, and is accompanied by bundling of microtubules. In contrast, VRS binding does not cause any appreciable stabilization nor any bundling. This difference between the two aminoacyl-tRNA synthetases may be due to the fact that KRS is dimeric, which ensures a twice larger concentration of positive charges on the same molecule and therefore increases the binding strength, compared to that of monomeric VRS. Given the size of the KRS monomer (68 kDa), it is also possible that KRS bridges two tubulin subunits on the microtubule, which could also enhance the stability of the polymer. Bundling of microtubules was also observed with τ 218–235 synthetic peptide and in this case too appears to be associated to microtubule stabilization. It was recently suggested (Chapin et al., 1991) that microtubule bundling in cells occurs as a two-step process involving stabilization of microtubules by any of a variety of unrelated treatments (overexpression of MAP-2, injection of Taxol or non-hydrolyzable analogues of GTP, etc.), followed by bridging that is mediated by an as yet unidentified bundling protein. The present results demonstrating microtubule stabilization and bundling by KRS or by the τ-2 (218–235) synthetic peptide, together with previous in vitro observations of microtubule bundling induced either by glyceraldehyde-3-phosphate dehydrogenase (Kumagai & Sakai, 1983; Huitorel & Pantaloni, 1985; Somers et al., 1990) or by subtilisin cleavage of the C-terminal region of tubulin (Sackett et al., 1985; Bhattacharyya et al., 1985; Peyrot et al., 1990), lead us to propose that microtubule bundling is a direct consequence of microtubule stabilization which can be achieved by a variety of treatments that lead to neutralization of the acidic C-terminal region. In other words, bundling would not necessitate the presence of an exogenous bundling factor and would simply result from charge attraction between the polymers. The fact that bundling is also observed in the presence of a non-hydrolyzable analogue of GTP (Wehland & Sandoval, 1983) interestingly suggests that the accessibility of the tubulin C-terminus region on microtubules may be modulated by the nature of bound nucleotide, a hypothesis that has recently been raised (Burns & Surridge, 1990). Within the above interpretation, the failure of VRS to bundle microtubules is due to insufficient stabilization. Alternatively, bundling by KRS might be linked to the dimeric structure of this protein, which would allow bridging of two microtubules bound to the cluster of positive charges on each subunit. However, this alternate explanation seems less plausible because it cannot comprehensively account for the bundling action of other stabilizing agents, in particular, τ-2 peptide.

Upon increasing the concentration of KRS or VRS, the stabilization of microtubules was reversed, and inhibition of polymerization was observed. A maximum stoichiometry of only 0.5 KRS and 0.2 VRS per assembled tubulin could be reached. A plausible explanation is that steric hindrance may prevent self-assembly of the 1:1 complexes of tubulin and KRS or VRS. Alternatively, the net charge of these complexes may be unfavorable for mutual interaction and subsequent self-assembly.

This behavior can be described by a thermodynamic model within which KRS and VRS bind to unpolymerized and polymerized tubulins with equilibrium association constants K_x and $K'_{x,app}$, respectively, and the value of $K'_{x,app}$ decreases with the fraction of polymerized tubulin with the ligand bound, $\bar{X}$, according to the equation (Tanford, 1961):

$$K'_{x,app} = K'_x e^{-f(\bar{X})}$$

which describes the binding of a ligand to identical sites that interact with one another. In the present case, $f(\bar{X})$ is an increasing function of $\bar{X}$; therefore, occupation of some sites on microtubules by KRS or VRS makes the binding at other sites more difficult. In a range of low values of $\bar{X}$, because $K'_x > K_x$, KRS has a higher affinity for microtubules than for dimeric tubulin and binding shifts the polymerization equilibrium toward the polymerized state of tubulin. As saturation of microtubules by KRS increases, $K'_{x,app}$ decreases and at some point becomes lower than K_x; then the polymerization equilibrium is shifted toward dimeric tubulin. In the case of VRS, the same model applies, but $K_x \simeq K'_x$, so that only destabilization of microtubules is observed as $\bar{X}$ increases.

Comparison of the binding of MAPS, KRS, or VRS and τ-2 synthetic peptide to unmodified or subtilisin-cleaved tubulin has been useful to probe the location of the binding site of MAPs in the C-terminal region of tubulin. Using radioactively labeled τ-2 synthetic peptide, we have shown evidence for 2 binding sites on both the intact $\alpha\beta$ and partially digested $\alpha\beta_S$-tubulin dimer and only 0.9 on $\alpha_S\beta_S$-tubulin. The finding of 2 binding sites/$\alpha\beta$-tubulin dimer agrees with the results of Maccioni et al. (1989), who found 1.85 and 1.74 binding sites/tubulin dimer for the synthetic polypeptides τ-1 (187–204) and τ-2 (218–235), respectively. The equilibrium dissociation constant we find here for τ-2 (K_D = 0.4 mM) is in good agreement with the value of 0.5 mM recently reported by Joly and Purich (1990) for the same peptide and the value of 0.18 mM reported by Aizawa et al. (1989) for the τ-1 peptide. The fact that only one of the two τ-2 binding sites is lost following proteolytic cleavage of α and β by subtilisin, with no change in affinity, suggests that subtilisin cleavage might occur at a position around 430–435 on α and β, thereby removing the peptide binding site on α- but not on β-tubulin. Our data therefore support the view (Bhattacharyya et al., 1985; De la Viña et al., 1988; Paschal et al., 1989) that subtilisin cleavage removes much less than the 4-kDa fragment from the C-terminus of tubulin, which was previously proposed (Serrano et al., 1984a,b). The subtilisin cleavage in the region of residues 430–435 on both tubulin subunits would be consistent with τ binding to the conserved sequences α[430–441] and β[422–434] previously proposed as part of the MAP binding sites (Maccioni et al., 1987, 1988; Rivas et al., 1989; Vera et al., 1988). Further support is brought by the observation that the binding of MAPs, KRS, or VRS to microtubules is weakened, yet not abolished, following subtilisin cleavage of tubulin. The C-terminal acidic amino acids remaining on $\alpha_S\beta_S$-tubulin are sufficient to allow MAP binding with reduced affinity, and this residual binding appears essentially electrostatic. All data convey the conclusion that the binding site of MAPs is not restricted to the extreme C-terminus that can be cleaved off by subtilisin, yet it includes it since its removal leads to a weakened binding. In particular, our results reconcile apparently discrepant reports, one by Rodionov et al. (1990), stating that subtilisin treatment of tubulin abolishes the binding of MAPs, the other by Vera et al. (1989) showing that MAPs are still bound to microtubules following removal of the extreme 1-kDa C-terminal portion of tubulin. Indeed, the present results show that MAPs cosediment with microtubules assembled from $\alpha_S\beta_S$-tubulin when a standard sedimentation assay is used (as used by Vera et al.), while they remain in the supernatant when microtubules are sedimented over a sucrose cushion according to Rodionov et al. (1990), a method that allows only the proteins tightly bound to microtubules to cosediment. Consequently, methods for quantitative measurement of binding parameters have to be designed in order to assess the effect of any modification of tubulin on MAP binding.

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