Separation of Tubulin Subunits under Nondenaturing Conditions†

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ABSTRACT: The dissociation and separation of the tubulin α- and β-subunits have been achieved by binding α-subunits to an immunoadsorbent gel and selectively inducing release of free β-subunits. The immunoadsorbent gel was prepared by coupling the monoclonal antibody YL$^1/_2$ to Sepharose 4B which specifically recognizes the C-terminal end of tyrosinated α-subunits. Extensive tubulin subunit dissociation and separation occurred in Tris buffer at neutral pH but was greatly enhanced at basic pHs (8.0−8.5). The binding of colchicine to heterodimeric tubulin resulted in a marked protection against dissociation. The dissociation of tubulin subunits was accompanied by loss of colchicine binding capacity, and ability to polymerize into microtubules. As shown by circular dichroism, loss of functional properties was not due to extensive denaturation of tubulin, as tubulin retained most of its secondary structure. Neither of the separated α- or β-subunits was able to bind colchicine, but functional tubulin that was able to bind colchicine could be reconstituted from the dissociated subunits by changing the buffer to a neutral mixture of Tris and Pipes. The yield of reconstitution, as estimated from kinetic measurements of colchicine binding capacity, amounted to about 25%. Such a yield can probably be improved with minor changes in experimental conditions. The quantitative dissociation of tubulin into separated "native" α- and β-subunits should provide a powerful tool for further studies on the properties of the individual tubulin subunits and the structure−function relationships of the tubulins.

Microtubules are involved in many cellular functions, including morphogenesis, mitosis, and transport of intracellular organelles (*1*). The architecture and polymerization dynamics of microtubules are central to their varied functions and are determined in large part by the properties of the tubulin dimer, the αβ-heterodimeric building block of the microtubule (*2*). The opposite ends of microtubules are structurally and kinetically distinct, due to the asymmetry of the tubulin heterodimer in the microtubule lattice (*1*, *3*). The α-chains of the tubulin dimer are exposed at one microtubule end; the β-chains are exposed at the other end. In cells, microtubules have uniform polarity with one end, the minus end, anchored at the centrosome and the opposite or plus end facing away from the centrosome (*4*, *5*). Such a uniform orientation is likely to be determined by differential interactions of α- and β-tubulins with centrosomal components (*6*, *7*). The asymmetry of the tubulin heterodimer must also be an important determinant for the directional movement of molecular motors and their cargos along microtubules (*8*−*14*). Microtubule dynamics are largely dependent on the GTPase activity of the tubulin molecule. In this reaction, GTP binds to an exchangeable site in β-tubulin (*15*). The hydrolysis reaction is triggered by the tubulin polymerization process through unknown mechanisms. Microtubule dynamics are regulated by the association of microtubules with regulatory proteins (MAPs)[1] (*16*−*22*). The assembly state of microtubules can also be dramatically modified through specific interactions of the tubulin molecules with various drugs (*23*−*27*). The precise location of

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[1] Abbreviations: CD, circular dichroism; IgG, immunoglobulin G; mAb, monoclonal antibody; MAP, microtubule-associated protein; STOP, stable tubule only polypeptide.

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the many MAPs and drug binding sites on tubulin has been difficult to determine.

A comprehensive understanding of the function of the tubulin dimer will involve understanding the specific roles of the individual α- and β-tubulin monomers. One approach that could facilitate such an understanding would involve separation of α- and β-tubulin into functional monomers. However, this has not yet been accomplished, and our knowledge of the molecular basis of tubulin function has been limited by the lack of a convenient procedure for obtaining homogeneous preparations of native α- and β-tubulin monomers.

Reversible dissociation of the αβ-tubulin heterodimer following dilution in Pipes-based buffer under nondenaturing conditions has been demonstrated previously using equilibrium centrifugation and fluorescence anisotropy (*28*−*34*). Tubulin dimers have also been shown to dissociate into α- and β-monomers after treatment with lactoperoxidase, which forms stable complexes with α- and β-tubulins (*35*). Such data show that tubulin subunit dissociation can occur under nondenaturing conditions. Despite these accomplishments, the physical separation of tubulin subunits to yield purified preparations of native α- and β-tubulin subunits has not been accomplished.

In this study, we have used an immunoaffinity procedure to achieve dissociation of tubulin hetrerodimers into individual α- and β-tubulin subunits. We find that α- and β-tubulin subunits can be readily separated in Tris-based buffers. Following dissociation to monomers in Tris buffer, tubulin loses its colchicine binding capacity but it retains most of its secondary structure, as determined by circular dichroism spectroscopy. Dissociated tubulin subunits can be reassociated to form dimers which regain their colchicine binding ability. These results establish a basis for the preparation of biochemical amounts of apparently native individual tubulin subunits and provide direct evidence that colchicine binding to tubulin requires the intact heterodimer.

MATERIALS AND METHODS

Tubulin Preparation. Tubulin was purified from fresh bovine brain by phosphocellulose column chromatography (Whatman P11, Clifton, NJ) as described previously (*36*). The purified tubulin was concentrated to ≈15 mg/mL by Centricon 30 ultrafiltration (Amicon, Danvers, MA) in PME buffer [100 mM Pipes [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 1 mM $MgCl_2$, and 1 mM EGTA [ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (pH 6.65)] containing 1 mM GTP and stored at −80 °C. Tubulin tyrosine ligase (TTL) was isolated by immunoaffinity chromatography as described by Wehland et al. (*37*), except that bovine brain was used as the source of the enzyme instead of porcine brain. Fully tyrosinated tubulin was affinity-purified from phosphocellulose-purified tubulin as described previously (*38*). We have found that D_2O is a powerful stabilizing agent for affinity-purified tyrosinated tubulin. This form of tubulin loses polymerization capacity upon freezing in a H_2O-based PME buffer, while it remains fully functional in a D_2O-based PME buffer. Thus, tyrosinated tubulin was concentrated to ≈5 mg/mL (Centricon 30) and transferred to PME buffer containing 1 mM GTP made with D_2O instead of H_2O, and the mixture was stored at −80 °C.

Immunoaffinity Gel Chromatography. Immunoaffinity chromatography was used for analysis of tubulin dissociation and isolation of separated tubulin subunits. $YL^1/_2$ IgG was purified from ascites fluid (*39*) and coupled to cyanogen bromide-activated Sepharose 4B (*40*). In control experiments, horse IgG (*39*), purified from serum, was used instead of $YL^1/_2$ IgG.

Colchicine Binding Assays. Colchicine binding to tubulin was assayed using [^{3}H]colchicine or by fluorescence (*41*). For the [^{3}H]colchicine binding assay, different concentrations of unlabeled colchicine containing [^{3}H]colchicine (2.5 μCi/mL) were mixed at 0 °C and then the mixtures incubated with the desired concentration of tubulin for 30 min at 30 °C. Aliquots (200 μL) were then mixed with 50 μL slurries of presedimented DEAE Sephadex A50 (to adsorb the tubulin with its bound colchicine) in PME buffer in 5 mL plastic tubes. All subsequent steps were carried out at 0−4 °C. Samples were incubated for 10 min with continuous shaking to ensure quantitative binding of the tubulin to the gel, after which they were centrifuged at 2350*g* for 4 min to pellet the gels. Supernatants were discarded, and the pellets containing the bound colchicine−tubulin complex were washed four times with 1 mL volumes of PME containing 10% DMSO. Pellets were incubated for 10 min with 500 μL of ethanol to solubilize the [^{3}H]colchicine, and 400 μL aliquots of the ethanol solutions were transferred to 10 mL of Readysafe (Beckman) scintillant for determination of radioactivity.

The fluorescence assay was used to measure the binding of colchicine to tubulin dimers. Tubulin solutions at various tubulin concentrations were incubated at 30 °C with continuous stirring in a 1 cm × 1 cm fluorescence cell. Colchicine (5×10^{-5} M) was then added, and emission spectra were collected using a MOS-200 optical system (Bio-Logic S. A., Claix, France). The monochromator was set at 367 nm, and an interferential filter at 436 nm was used.

Interpretation of Fluorescence Plots. Fluorescence plots were used to compare the concentrations of active tubulin dimers at the onset and at the end of reconstitution experiments (see the text). The kinetics of fluorescence changes were consistent with the simple bimolecular binding scheme between tubulin T and colchicine C:

$$C + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CT$$

Under the experimental conditions of reconstitution experiments, the concentration of free colchicine C remained almost constant, and this reaction followed pseudo-first-order kinetics with the rate law

$$[CT] = k_1C_0T_0/(k_1C_0 + k_{-1})[1 - e^{-(k_1C_0+k_{-1})t}]$$

The plateau at $t = \infty$ is $k_1C_0T_0/(k_1C_0 + k_{-1})$, and the initial slope at $t = 0$ is $k_1C_0T_0$. For a fixed total colchicine concentration C_0, both the initial slope and the plateau are proportional to the tubulin concentration, T_0. Reconstitution experiments are based on this proportionality between tubulin concentrations, initial slopes, and plateaus.

Circular Dichroism (CD) Spectra. CD spectra were recorded on a CD6 Jobin-Yvon spectropolarimeter. Spectra were routinely recorded between 178 and 260 nm with an

integration time constant of 15 s and a spacing of 1.5 nm. All measurements were made at 4 °C using a quartz cell with a 0.01 mm optical path.

Miscellaneous Procedures. Rapid desalting of tubulin samples was performed by a centrifugation−filtration method with Bio-Gel P-6 (*42*). SDS−PAGE (*43*) was performed using 7.5% acrylamide resolving gels, and protein bands were visualized with Coomassie Brilliant Blue. A turbidimetric assay of microtubule assembly was performed as described by Pirollet et al. (*44*). Tubulin concentrations were determined spectrophotometrically using an extinction coefficient of 1 mL mg^{-1} cm^{-1} at 280 nm.

Chemicals and Reagents. Radiolabeled colchicine ([*ring C,methoxy*-^{3}H], 70 Ci/mmol; 1 Ci = 37 Gbq) was obtained from New England Nuclear (Boston, MA). D_2O was from Cambridge Isotope Laboratories (Cambridge, U.K.). Nucleotides were obtained from Boehringer Mannheim (Indianapolis, IN) and Bio-Gel P-6 and Tris from Bio-Rad Laboratories (Richmond, CA); cyanogen bromide was obtained from Pierce (Rockford, IL) and imidazole from Prolabo (Rhône-Poulenc, France). DEAE Sephadex A50 and Sepharose 4B were from Pharmacia (Uppsala, Sweden), and unlabeled colchicine was obtained from Merck (Darmstadt, Germany). Monoclonal anti-tyrosinated α-tubulin (clone $YL^1/_2$) was a generous gift from J. V. Kilmartin (MRC Laboratories, Cambridge, U.K.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Measurement of Tubulin Heterodimer Dissociation by Batchwise Specific Immunoadsorption of Tyrosinated α-Subunits. To measure the dissociation and separation of α- and β-tubulin subunits, an immunoadsorption assay specific for the α-subunits was devised as follows. The immunoadsorbent gel was prepared by covalently coupling Sepharose 4B to monoclonal antibody $YL^1/_2$. This antibody reacts specifically with the carboxy terminus of tyrosinated α-tubulin and shows no cross reactivity with β-tubulin. In the absence of any dissociation of tubulin, tyrosinated dimers remain bound to the immunoadsorbent gel by their tyrosinated α-subunits. If the heterodimer dissociates, the tyrosinated α-subunits bind to the immunoadsorbent gel, and β-subunits remain free in solution. Rapid sedimentation will therefore separate the free β-subunits in solution from those bound to α-subunits on the immunoadsorbent gel. Purified heterodimeric tubulin was incubated for 30 min at a final protein concentration of 2 μM in various buffers in the presence of a mAb $YL^1/_2$−Sepharose 4B immunoadsorbing gel. After sedimentation, the protein content of the supernatant was analyzed by SDS−PAGE, and the content of β-subunits in the supernatant revealed the extent of heterodimer dissociation.

The first buffer used to search for dissociation was PME buffer, since several reports indicated that reversible dissociation of tubulin can occur in this buffer (*28*−*34*). However, when tubulin was incubated for 30 min at a final concentration of 2 μM in the presence of the mAb $YL^1/_2$−Sepharose 4B gel, the supernatant fraction contained small and equal amounts of the α- and β-subunits of tubulin, showing no detectable accumulation of free β-tubulin (Figure 1). Similar results were obtained at tubulin concentrations ranging from 0.5 to 2 μM and for incubation times ranging from 30 to 120 min. Control experiments performed with nonspecific IgGs coupled to Sepharose 4B showed that the supernatant contained all of the initial tubulin αβ-heterodimer regardless of the tubulin concentration and the incubation time. These results suggested that both tubulin subunits

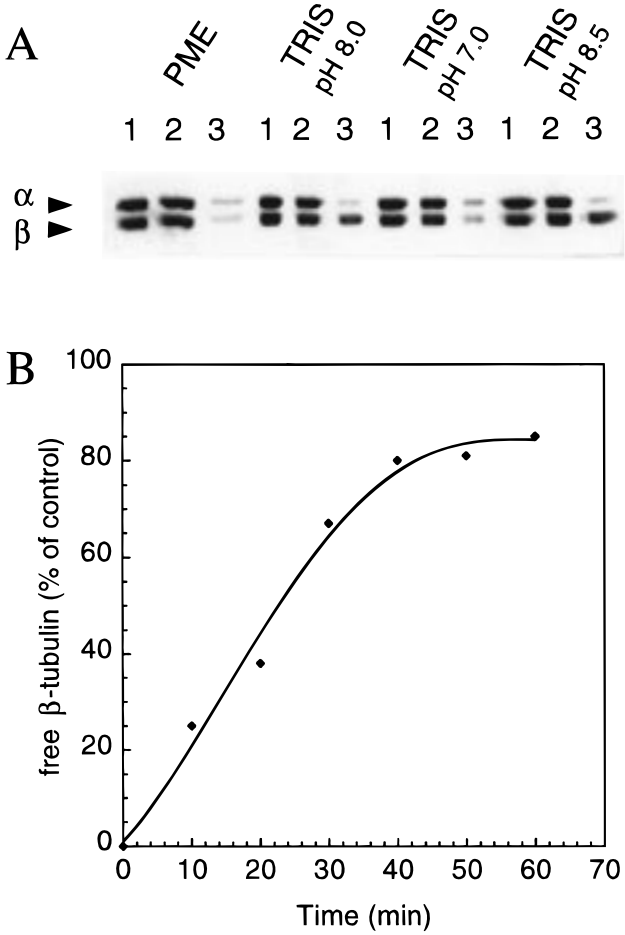

FIGURE 1: Analysis of tubulin dissociation in various buffers. (A) Tyrosinated tubulin (8×10^{-5} M in PME buffer) was diluted 40-fold in various buffers, as indicated. Tubulin solutions (2 μM) were incubated for 30 min at 0 °C. Tubulin solutions were then mixed with packed Sepharose 4B gel, containing either coupled mAb $YL^1/_2$ or horse IgGs, at a ratio of 50 μL of tubulin solution per 20 μL of immunoaffinity gel. The suspensions were kept at 4 °C for 30 min under continuous shaking. The gel was further pelleted by centrifugation (600*g* for 3 min at 4 °C). For each buffer condition, aliquots (20 μL) of the supernatants and of control tubulin were run into 7.5% SDS gels and analyzed for relative amounts of α- and β-tubulin. Four experimental series, corresponding to the indicated buffer conditions, are shown. Each experimental series is shown in three lanes: lane 1, control tubulin; lane 2, supernatant obtained following incubation of tubulin solutions with horse IgGs Sepharose; and lane 3, supernatant obtained following incubation of tubulin solutions with mAb $YL^1/_2$−Sepharose. (B) Time course of tubulin dissociation in Tris buffer at pH 8.0. Tubulin dimers were incubated in Tris buffer at pH 8.0 as in panel A. At the indicated time points, tubulin solutions were mixed with packed Sepharose 4B gel containing coupled mAb $YL^1/_2$ and the mixtures incubated for 5 min at 4 °C under continuous shaking. The gel was then pelleted, and aliquots of the supernatants were run into SDS gels as in panel A. In control experiments, tubulin in Tris buffer at pH 8.0 was mixed with Sepharose 4B gel containing coupled horse IgGs instead of mAb $YL^1/_2$, and supernatants were analyzed on SDS gels as above. The amounts of β-tubulin present in the supernatants were quantified by gel scanning. Results are expressed as percentages of β-tubulin in the supernatants of mAb $YL^1/_2$-treated samples. The 100% value corresponds to the β-tubulin signal observed in controls.

remained bound to the mAb YL$^1/_2$−Sepharose 4B gel in PME buffer, without any significant separation of α- and β-tubulin. The values of 0.1−1 μM reported for the equilibrium dissociation constant K_d of tubulin in PEM buffer (*28*−*34*) would have predicted a tubulin dissociation of >50%, with 0.5 μM tubulin. The absence of detectable free β-tubulin in any of the supernatants suggests that the K_d under our conditions is lower than expected from previous work. This lower K_d could be due to slight differences in experimental conditions. It could also be due to the fact that we measure the dissociation of αβ-tubulin bound to the mAb YL$^1/_2$−Sepharose 4B gel, and the possibility that the interaction between the α- and β-subunits is stabilized by the binding of the mAb YL$^1/_2$ to the tyrosinated α-subunit cannot be excluded.

Using the same procedure of batch immunoadsorption and centrifugation, we have looked for the appearance of free β-tubulin in supernatants after incubation of tubulin in other buffers. No evidence for dissociation of the αβ-dimer was observed in phosphate, imidazole, or Hepes buffers at pH 7.0 or 8.0. Only Tris buffer appeared to promote some dissociation of tubulin. After incubation of tubulin for 30 min in 0.2 M Tris-HCl buffer at pH 8.0, >60% of β-tubulin was present in the supernatant (Figure 1A,B). After 60 min, >80% of the β-tubulin was in the supernatant, indicating extensive dissociation of tubulin dimers (Figure 1B). The extent of dissociation decreased with decreasing concentrations of Tris buffer at pH 8.0 (not shown). Dissociation of the tubulin increased when the pH was raised to 8.5 in 0.2 M Tris-HCl buffer, whereas no dissociation took place in the same buffer at pH 7.0 (Figure 1). The stability of the αβ-tubulin heterodimer was therefore sensitive to the nature and concentration of the buffer and to the pH (Figure 1).

Chromatographic Measurement of Tubulin Heterodimer Dissociation by Specific Immunoadsorption of Tyrosinated α-Subunits. To separate α-and β-tubulin subunits more efficiently than with batchwise absorption, we used mAb YL$^1/_2$ coupled to Sepharose 4B in an immunoaffinity column. Tyrosinated αβ-tubulin dimers in PME buffer were loaded onto columns of mAb YL$^1/_2$−Sepharose 4B and eluted with various other buffers. As expected from batchwise experiments, the protein fraction that eluted with 0.2 M Tris buffer at pH 8.0 was mainly β-tubulin. Further elution with the same buffer supplemented with 0.8 M NaCl yielded a protein fraction mainly composed of α-tubulin subunits. The yields of recovered protein were 45 and 35% of the total initial load for α- and β-tubulin, respectively (Figure 2). Similar experiments, using 0.2 M Tris elution buffer, were also performed at pH 8.5 and 7.0. A pH of 8.5 sharpened the β-tubulin peak and increased its height (Figure 2). A pH of 7.0 resulted in a small broad β-tubulin peak (Figure 2). This chromatographic evidence for a partial dissociation of tubulin in Tris buffer at pH 7.0 may appear surprising since no dissociation could be detected in batchwise experiments with the same buffer (see above). The same apparent disagreement between an absence of dissociation with a batchwise procedure and significant dissociation upon chromatography was also observed with imidazole buffer at slightly alkaline pH (not shown). There is, however, a marked difference between the two procedures. During chromatography, the putative equilibrium between intact αβ-dimers and dissociated subunits could shift toward dissociation by the continuous removal of the free β-tubulin. Thus, the extent of dissociation would be expected to increase with the time taken by the chromatographic separation. It was indeed observed that the extent of tubulin dissociation was strongly dependent on the flow rate of elution. In neutral Tris, a rapid elution within 30 min of loading dimeric tubulin on the column did not result in any detectable dissociation (not shown). Some dissociation of tubulin could only be monitored when elution was slow (Figure 2). It took an elution time of 1 h to recover about 25% of total β-tubulin as isolated free β-subunits. The dimeric tubulin bound to the column could be largely dissociated by extensive washing with 0.2 M Tris buffer at pH 7.0, as shown by the large excess of isolated α-subunits present in the gel-bound fraction that is further eluted with 0.8 M NaCl (see the bottom SDS−PAGE profiles in Figure 2B).

We have previously reported that αβ-tubulin could be loaded on mAb YL$^1/_2$ affinity columns for long periods of time and extensively washed with large volumes of PME buffer without any detectable alteration in the composition or biochemical properties of its dimeric structure (*38*). We have repeated such experiments and found no dissociation of αβ-tubulin during affinity chromatography in PME buffer. Similarly, no dissociation of tubulin was observed using this chromatographic procedure with Hepes or phosphate buffers, at neutral or slightly alkaline pH.

Influence of Tris Buffer on the Functional Properties of Dimeric αβ-Tubulin. To rule out the possibility that tubulin was dissociated by Tris buffer because the protein was denaturing, we tested whether Tris buffer by itself had a marked influence on the functional properties of dimeric tubulin. As dissociation of tubulin was very slow in 0.2 M Tris buffer at pH 7.0 (see above), some functional properties could be determined in this buffer. The two functional properties examined were the ability to bind colchicine and the ability to polymerize into microtubules. Colchicine binding was measured immediately after dilution of dimeric tubulin into Tris or PME buffers. The amount of colchicine bound and the affinity of tubulin for colchicine were similar in the two buffers (compare panels A and B of Figure 3). Colchicine binding was also measured after incubating tubulin in Tris or PME buffers for a given time. Over a period of 60 min, the extent of colchicine binding remained constant in PME buffer and decreased slowly by about 15% in Tris buffer (Figure 3C). The loss of colchicine binding occurred more rapidly as the pH was raised to 8.0 or 8.5 (Figure 3C). This pH dependence was consistent with the increased release of isolated β-subunits at alkaline pH (Figure 1). Furthermore, at pH 8, the time course for loss of colchicine binding activity was similar to the time course for dimer dissociation (Figures 1B and 3C). These data indicate that loss of colchicine binding occurs mainly as a result of dimer dissociation.

Native dimeric αβ-tubulin was unable to polymerize into microtubules in Tris buffer. However, tubulin largely retained its ability to polymerize into microtubules when incubated for at least 1 h in 0.2 M Tris buffer at pH 7.0, after transfer into PME buffer by gel filtration (Figure 3D). This suggests that a long incubation of tubulin in Tris buffer at neutral pH had little deleterious effect on the native structure of the tubulin dimer.

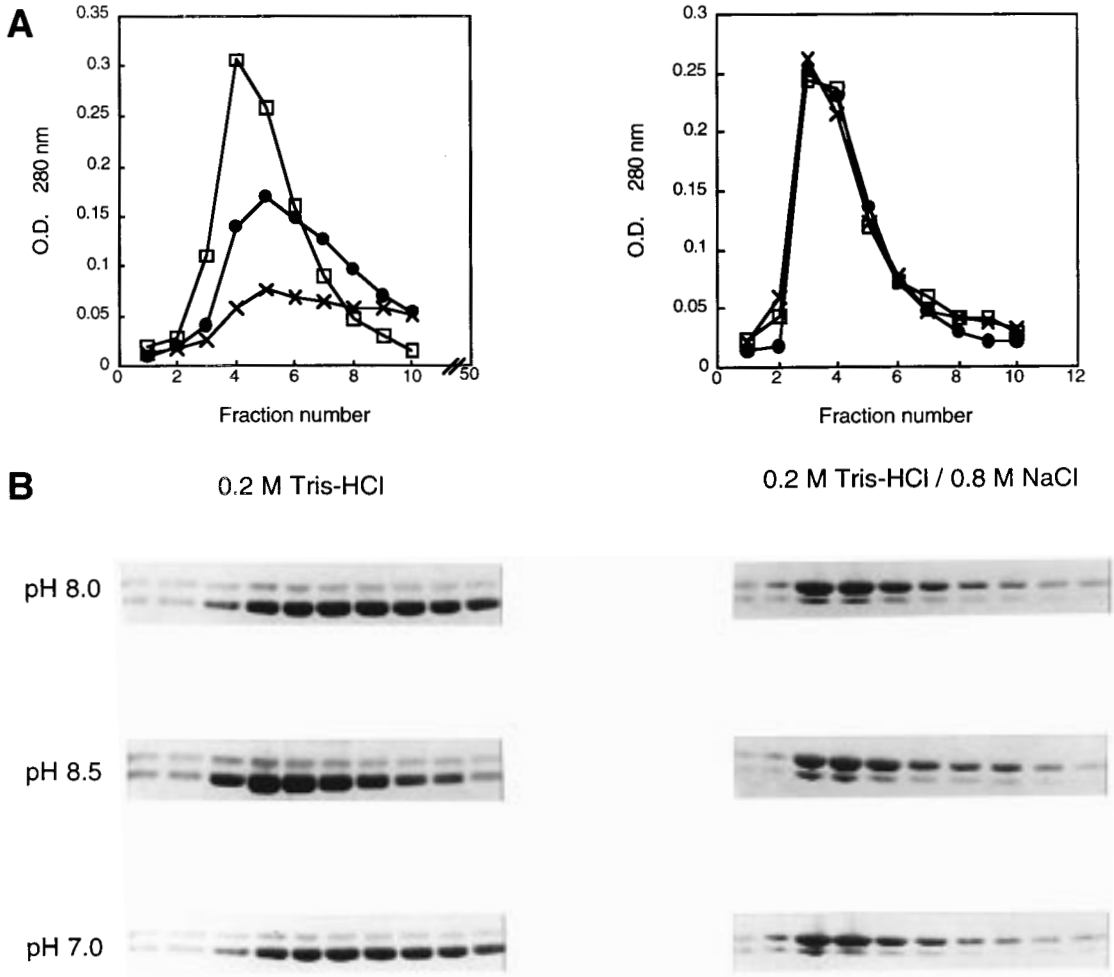

FIGURE 2: Analysis of tubulin subunit separation during immunoaffinity column chromatography in various Tris-based buffers. Aliquots of tyrosinated tubulin (40 μM in PME buffer) were loaded onto three $YL^1/_2$ immunoaffinity columns at a tubulin concentration of 2 mg/mL of gel. The columns were further washed with 30 column volumes of PME buffer containing 0.1 M NaCl. Columns were further eluted with 0.2 M Tris-HCl buffers, at either pH 8.0, 8.5, or 7.0. Columns were eluted stepwise with 5 column volumes of 0.2 M Tris-HCl buffer. Fractions (half a column volume) were collected and analyzed for protein content by OD_{280} measurements and for protein composition by SDS−PAGE analysis. The elution time was about 15 min in the case of the Tris experiments at pH 8.0 and 8.5. At such an elution rate, no tubulin dissociation occurred in Tris experiments at pH 7.0 (not shown). In the experiment at pH 7.0, the elution rate was adjusted to 1 column volume/10 min. Columns were further washed with 10 column volumes of 0.2 M Tris-HCl buffer containing 0.05 M NaCl prior to elution with 5 column volumes of 0.2 M Tris-HCl buffer containing 0.8 M NaCl. Elution fractions (half a column volume) were collected and analyzed for protein content and protein composition as described above. (A) OD_{280} measurements: (left) the 0.2 M Tris-HCl elution step, (right) the 0.2 M Tris-HCl, 0.8 M NaCl elution step, (□) elution at pH 8.5, (●) elution at pH 8.0, and (×) elution at pH 7.0. (B) SDS−PAGE analysis of protein fractions as identified in the figure.

We have also separated the tubulin subunits by chromatography on the mAb $YL^1/_2$−Sepharose 4B gel and monitored the elution profile by the ability to bind colchicine (Figure 4A) and by SDS−PAGE (Figure 4B). After dimeric tubulin was loaded onto the immunoaffinity column, a slow elution was performed using 0.2 M Tris buffer at pH 7.0. The first fractions contained only small and equivalent amounts of α- and β-tubulin, indicating that the initial tubulin could contain a minor contamination by a $\alpha\beta$-tubulin dimer that was not or was poorly retained by the affinity gel. This contaminant may be a de- or untyrosinated dimer since mAb $YL^1/_2$ binds specifically tyrosinated α-tubulin. As seen from the intensity of the band of α-tubulin, the amount of this dimeric contaminant decreased in the subsequent fractions that contained the peak of isolated β-subunits (Figure 4B). The concentration of this dimeric contaminant as determined by gel scanning was about 0.1−0.15 μM, compared to 2 μM total initial $\alpha\beta$-tubulin.

Colchicine binding assays showed that a species able to bind colchicine was present in the first fractions and disappeared progressively from the subsequent fractions in which the excess of isolated β-subunits appeared (Figure 4A). A maximum concentration of 0.08−0.1 μM was found for bound colchicine, together with an apparent dissociation constant of ca. 50 μM, close to that of native $\alpha\beta$-tubulin (data not shown). These results suggested that isolated β-tubulin subunits had no detectable colchicine binding activity in our assay conditions and that all the colchicine binding activity detected in the first fractions was due to the dimeric contaminant. We also found that a very low amount of colchicine could be bound to the tubulin that remained attached to the affinity gel after dissociation and elution of the β-subunits, but this colchicine binding activity was probably due to residual dimeric contaminants (not shown). The conclusion is thus that isolated tubulin subunits are unable to bind colchicine and that the loss of colchicine

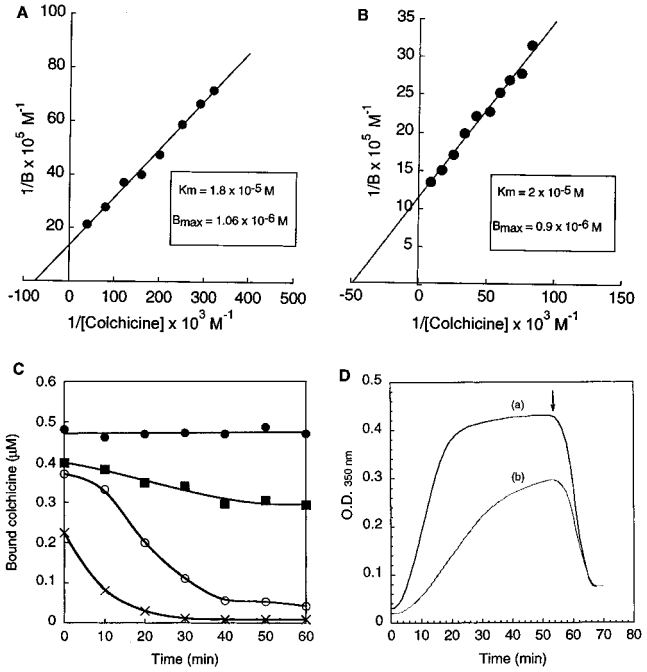

FIGURE 3: Effect of Tris-based buffers on the functional status of tubulin, at different pHs. (A) Lineweaver−Burk analysis of tubulin−colchicine interaction in PME buffer. Tubulin (2 μM in PME buffer) was incubated with radiolabeled colchicine in the presence of various concentrations of unlabeled colchicine, as indicated. Colchicine binding was further assayed as described in Materials and Methods. (B) Same as panel A, except that tubulin was placed in neutral 0.2 M Tris-HCl buffer. (C) Time course analysis of the effect of Tris-based buffers at different pHs on the colchicine binding activity of tubulin. Tubulin (2 μM) was incubated at 0 °C in PME buffer or in 0.2 M Tris-HCl buffers at various pHs. At the indicated time points, the various tubulin solutions were incubated with radiolabeled colchicine to determine the remaining colchicine binding activity, as described in Materials and Methods. The colchicine concentration was 5×10^{-5} M: (●) PME buffer, (■) Tris buffer at pH 7.0, (○) Tris buffer at pH 8.0, and (×) Tris buffer at pH 8.5. (D) Effect of neutral Tris buffer on the tubulin capacity to polymerize. Tubulin (50 μM) was incubated for 60 min on ice either in PME buffer (a) or in neutral Tris buffer (b). Tris-treated tubulin was further exchanged in PME buffer, and samples were adjusted to 10% glycerol, 5% DMSO, and 10 mM $MgCl_2$. The final tubulin concentration was 40 μM. Tubulin assembly was monitored using OD_{350} turbidity measurements as described in Materials and Methods. When indicated (arrow), the temperature was decreased to 4 °C.

binding ability in 0.2 M Tris buffer is caused by dissociation of the $\alpha\beta$-dimer.

Incubation of tubulin in Tris buffer at pH 8.0 or 8.5 resulted in rapid loss in tubulin's ability to polymerize into microtubules (not shown) and the ability to bind colchicine, this loss being faster at pH 8.5 than at pH 8.0 (Figure 3C). Colchicine binding was lost too rapidly to be compared to the release of free β-tubulin measured by affinity chromatography, as done above for pH 7.0, but it is probable that this loss also reflected tubulin dissociation. The transfer of tubulin from alkaline Tris buffer back into PME buffer was not followed by a significant immediate regaining of colchicine binding. This indicated that dissociation in Tris buffer could be greatly accelerated by raising the pH and that dissociation cannot easily be reversed. This lack of reversibility could be due either to an intrinsic slowness of the reassociation of dissociated tubulin subunits or to an extensive denaturation of tubulin by alkaline Tris buffer. It

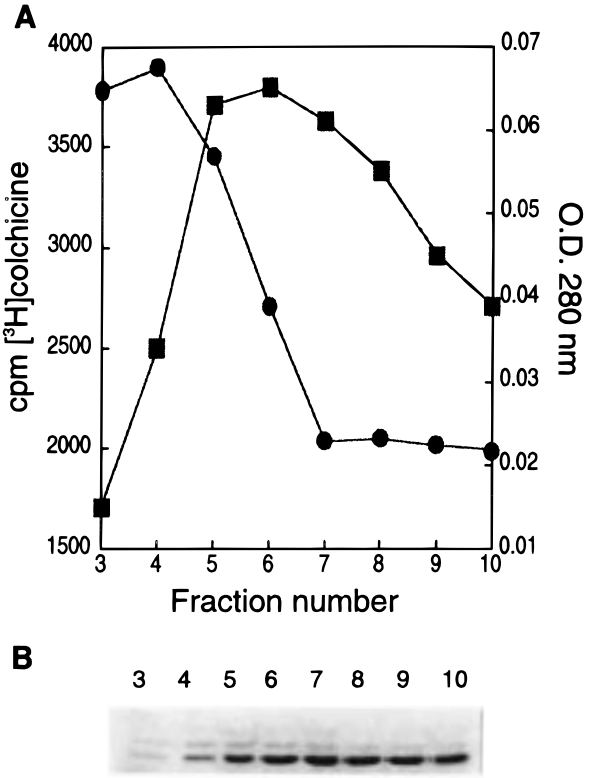

FIGURE 4: Analysis of colchicine binding activity in column fractions during immunoaffinity chromatography of tubulin in neutral Tris buffer. Tubulin was loaded onto a $YL^1/_2$ affinity column, and the column was further eluted in neutral Tris buffer, as described in the legend of Figure 2. The fractions corresponding to the first 0.2 M Tris-HCl elution step were analyzed for protein content, colchicine binding activity, and protein composition. To minimize possible protein denaturation prior to colchicine binding assays, fractions were eluted in tubes containing radiolabeled colchicine and immediately assayed for colchicine binding activity. (A) (■) Protein content estimated by OD_{280} measurements and (●) colchicine binding activity measured using [^{3}H]colchicine assays. (B) SDS−PAGE analysis of protein fractions.

was therefore important to compare the structural states of dimeric $\alpha\beta$-tubulin and of the stoichiometric mixture of α- and β-subunits obtained after dissociation in alkaline Tris buffer.

Conservation of Tubulin Secondary Structure upon Dissociation into Separated α- *and* β-*Subunits.* Circular dichroism (CD) was used to compare the conformational states of tubulin as a dimer and as a mixture of dissociated α- and β-subunits. The far-UV wavelength range between 250 and 185 nm was chosen because spectra in this range are sensitive to changes in secondary structure and are thus a good index for extensive denaturation. CD spectra in the near-UV wavelength range are sensitive to minor structural changes that involve aromatic residues, notably tryptophans, and are poor indicators of overall denaturation. Between 250 and 185 nm, the CD spectra of tubulin in PME and in Tris buffers at pH 7.0 could be superimposed (not shown), showing that the buffer had no influence on the CD spectrum of dimeric tubulin. The CD spectra of tubulin in Tris buffer showed a small difference between pH 7.0 and 8.0. At pH 7.0, the trough at 210−220 nm was slightly deeper and the peak at 190 nm slightly smaller than at pH 8.0 (Figure 5), but both spectra corresponded to a well-folded protein. The changes observed upon raising the pH from 7.0 to 8.0 were not sufficiently large to be interpreted in terms of a decrease in

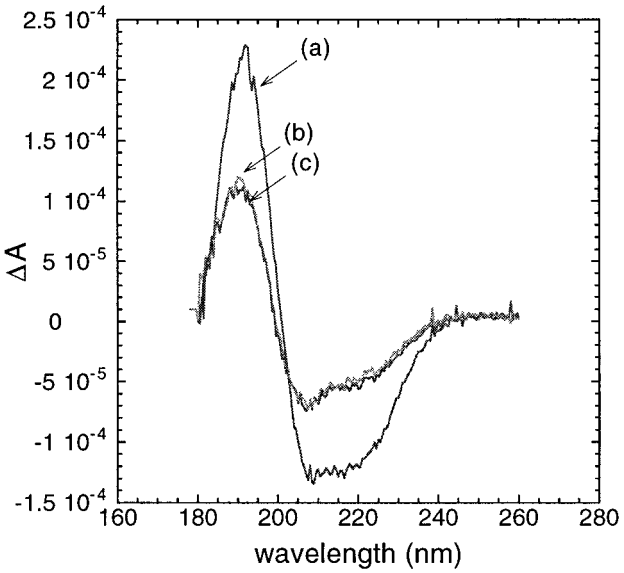

FIGURE 5: Comparison of the CD spectra of intact and dissociated tubulin dimers. CD spectra of 20 μM tubulin solutions placed in different buffers. (a) Pure tubulin was diluted in 0.2 M Tris-HCl buffer at pH 7.0, and the CD spectrum was immediately recorded. (b) Pure tubulin was diluted in 0.2 M Tris-HCl buffer at pH 8.0, and the CD spectrum was immediately recorded. (c) Pure tubulin was diluted in 0.2 M Tris-HCl buffer at pH 8.0 and incubated on ice for 30 min. Then, the CD spectrum was recorded.

the content of β-structure and an increase in α-helix. Remarkably, the spectra in Tris buffer at pH 8.0 did not show any time-dependent changes, and the same spectra were obtained immediately upon dilution of tubulin into Tris buffer at pH 8.0 (Figure 5) and 30 min later, at a time when tubulin has lost more than two-thirds of its colchicine binding capacity (Figure 3C). The CD spectrum was therefore insensitive to the transition from the dimeric state of tubulin into a largely dissociated one. Although CD spectra are not sufficient to exclude denaturation, such a similarity between the spectra of dimeric and dissociated tubulin suggests that dissociation in itself was not accompanied by any appreciable denaturation or major change in secondary structure.

In Tris Buffer, the Dimeric State of Tubulin Is Stabilized by the Binding of Colchicine. The preceding results strongly suggest that Tris buffer induces tubulin subunit dissociation in the absence of obvious tubulin denaturation. If this is correct, one would expect tubulin dissociation in Tris buffer to be impeded by the binding of colchicine, a drug known to stabilize the dimeric state of tubulin (*29*, *30*, *33*, *34*, *45*−*47*).

To test this possibility, we compared the rate of colchicine loss from preformed tubulin−colchicine complexes in Tris buffer (Figure 6A) with the rate of loss of colchicine binding activity of tubulin dimers under similar buffer conditions (Figure 3C). We also checked the tubulin subunit dissociation from preformed tubulin−colchicine complexes by measuring the release of free β-subunits from an affinity column loaded with such complexes and eluted with Tris buffer. The tubulin−colchicine complexes were first formed in PME buffer and subsequently transferred to Tris buffer at neutral or alkaline pH. The residual colchicine binding was then measured for 1 h. No significant loss of colchicine from tubulin−colchicine complexes was observed in Tris buffer at pH 7.0. In Tris buffer at alkaline pH, colchicine dissociated from tubulin at a rate that was markedly pH-dependent; the ratio of residual bound colchicine after 1 h in Tris buffer to the total bound colchicine at time zero exceeded 50% at pH 8.0 and was about 20% at pH 8.5 (Figure 6A). Comparison between Figures 6A and 3C showed that the loss of colchicine binding activity, and presumably the dissociation of tubulin subunits, were markedly slower for tubulin−colchicine complexes than for unliganded tubulin.

The subunit dissociation of tubulin in the presence of bound colchicine was also monitored by measuring the

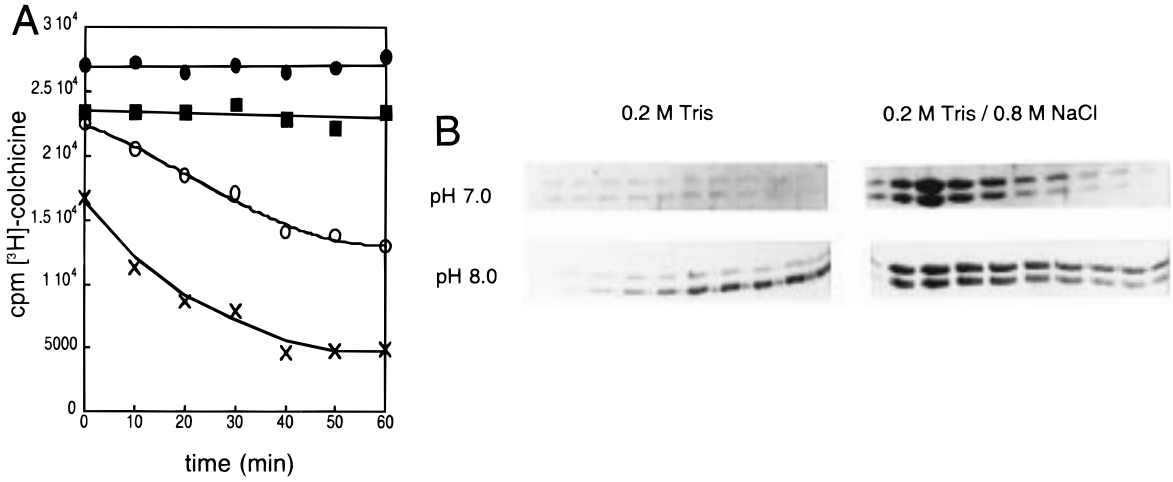

FIGURE 6: Effect of colchicine binding on the dissociation of tubulin dimers. (A) Time course analysis of tubulin−colchicine complex dissociation in various buffers. Tubulin (80 μM in PME buffer) was incubated for 30 min at 30 °C with radiolabeled colchicine in the presence of 0.4 mM unlabeled colchicine. Aliquots of the tubulin−colchicine solutions were then diluted 40-fold either in PME buffer or in 0.2 M Tris-HCl buffers at various pHs and further incubated on ice. At the indicated time points, aliquots of the various tubulin−colchicine solutions were removed and assayed for colchicine binding activity: (●) incubation in PME buffer, (■) incubation in Tris buffer at pH 7.0, (○) incubation in Tris at pH 8.0, and (×) incubation in Tris at pH 8.5. (B) Analysis of tubulin subunit separation during affinity chromatography of tubulin−colchicine complexes. Tubulin (40 μM in PME buffer) was incubated for 30 min at 30 °C with 0.4 mM colchicine. Aliquots of the tubulin−colchicine solution were loaded onto two mAb YL$^1/_2$ affinity columns. Columns were further washed and eluted stepwise as described in the legend of Figure 2. Elution steps were performed with Tris-based buffers at either pH 7.0 or 8.0, as indicated. The protein composition of each elution fraction was analyzed on 7.5% SDS gels.

Table 1: Values of Initial Slopes and Maximal Fluorescence Levels during Reconstitution Experiments[a]

	a	b	c	d	e	f	g	h
initial slope (mV/s)	6.29	2.76 (44)	1.27 (20)	1.20 (19)	1.06 (17)	6.68	0 (0)	0 (0)
plateau (mV)	3523	1451 (41)	1408 (40)	800 (23)	618 (18)	3700	555 (15)	169 (5)

[a] Initial slopes of fluorescence plots were determined as the slopes of the fluorescence vs time regression lines, during the first 60 s of fluorescence measurements. Incubation conditions described for the corresponding lanes in Figure 7. In parentheses are the percentage of control values.

release of β-subunits from tubulin−colchicine complexes using affinity chromatography as described above (Figure 2). The preformed tubulin−colchicine complexes in PME buffer were loaded onto the mAbYL$^1/_2$−Sepharose 4B affinity column, and elution was then carried out with 0.2 M Tris buffer at either neutral or alkaline pH. At pH 7.0, no release of isolated β-subunit could be observed by SDS−PAGE, showing that the tubulin−colchicine complex bound to mAb YL$^1/_2$ was stable and did not dissociate (Figure 6B). Some dissociation took place at pH 8.0, but much less free β-subunit was released from the tubulin−colchicine complex than from unliganded tubulin (compare Figures 6B and 2B). In some experiments, tubulin−colchicine complexes were formed in the presence of radiolabeled colchicine to test the possibility that colchicine remained bound to one of the two tubulin subunits following dimer dissociation. Such persistent binding was not observed (data not shown). These results suggested that dissociation of the tubulin−colchicine complex was slower than that of unliganded tubulin and that dimer dissociation also caused dissociation of colchicine from both tubulin subunits. The finding that colchicine increased the lifetime of $\alpha\beta$-dimers in alkaline Tris buffer confirmed that tubulin dissociation in this buffer was not caused by tubulin denaturation.

Reassociation of Dissociated α- and β-Tubulin Subunits into Active Dimers. It was important to test the reversibility of tubulin dissociation by reconstitution of dimeric tubulin from separated subunits. Such reconstitution would confirm that the separated α- and β-subunits retained sufficient native structure to reassociate. In an ideal reassociation experiment, α- and β-tubulin subunits should have been first separated by affinity chromatography in alkaline Tris buffer and then mixed again into neutral PME buffer. The formation of functional $\alpha\beta$-dimers could have been monitored by both colchicine binding activity and the ability to polymerize into normal microtubules. Such an experiment would involve (i) the successive elutions of β- and α-tubulin from affinity columns by low and high salt (Figure 2), (ii) the concentration of separated tubulin subunits, and (iii) the transfer from Tris into PME buffer by dilution or gel filtration. All attempts to achieve steps (ii) and (iii) have been unsuccessful. Concentrating individual α- or β-subunits led to their aggregation, and gel filtration resulted in a marked loss of protein, probably due to aggregation on the column. Before initiating a systematic search for proper conditions (e.g., buffers, membranes, resins, solvent additives, and ultrafiltration devices), we looked for simple experimental procedures to test whether dissociated tubulin could indeed reassociate into native $\alpha\beta$-dimers.

In the preceding sections, it is shown that colchicine binding is a specific capacity of only the dimeric state of tubulin, not of isolated α- and β-subunits or of an equimolar mixture of α- and β-subunits. Consequently, any regain of colchicine binding should reflect the reappearance of a functional dimeric structure. Therefore, we monitored the reappearance of colchicine binding ability of tubulin that had been first dissociated into its subunits. Colchicine binding was measured by fluorescence. It can be shown (see Materials and Methods) that, on fluorescence plots, for a fixed colchicine concentration, both the initial slope and the plateau are proportional to the tubulin concentration. Such proportionality is illustrated Figure 7 and Table 1. Curve a shows a control fluorescence plot, determined at a 2 μM tubulin concentration under nondissociating buffer conditions. Curves b and d show similar fluorescence plots determined at 0.8 and 0.4 μM tubulin concentrations, respectively. Control values for initial slopes and plateaus were determined on curve a. The corresponding values and their ratio to control values were determined on curves b and d. The results showed that these ratios were consistent with the ratios of tubulin concentrations (Table 1). For instance, at 0.4 μM tubulin (20% of the control tubulin concentration), the ratio of the initial slope to that of the control was 19% and the ratio of the plateau to that of the control was 23% (Table 1).

These data form the basis for quantitative reconstitution experiments. In successful reconstitution experiments, the concentration of active dimers increases with time and therefore the ratio of the plateau to that of the control is significantly higher than the corresponding ratio for the initial slope.

Such a successful reconstitution experiment is shown in curve c of Figure 7. Dissociation was achieved with a 30 min incubation of 2.2 μM tubulin in 0.2 M Tris buffer at pH 8.0 (Figure 7A). Reassociation was triggered by addition of a mixture, producing a final composition of 2 μM tubulin, 100 mM Pipes, 180 mM Tris, 1 mM DTT, 1 mM EGTA, 2 mM $MgCl_2$, and 1 mM GTP, at a final pH of 7.0, in the presence of 50 μM colchicine. The initial slope of the fluorescence plot amounted to 20% of the control, showing that dimer dissociation was approximately 80%. This was consistent with the results of the batchwise assays of tubulin dissociation shown Figure 1 and with the loss of more than two-thirds of the colchicine binding observed above under similar conditions (Figure 3C). However, the plateau reached after 2 h represented 40% of the control. This showed that the amount of tubulin able to bind colchicine had doubled during the measurement and indicated that about 25% of the initially dissociated tubulin subunits had reassociated into functional dimers.

When tubulin was dissociated at pH 8.5 instead of at pH 8.0 (Figure 7B), the initial slope was very small (Table 1), again consistent with the extensive tubulin dissociation shown by the batchwise technique (Figure 1) and with a loss of more than 95% of colchicine binding under these conditions (Figure 3C). After such a complete dissociation,

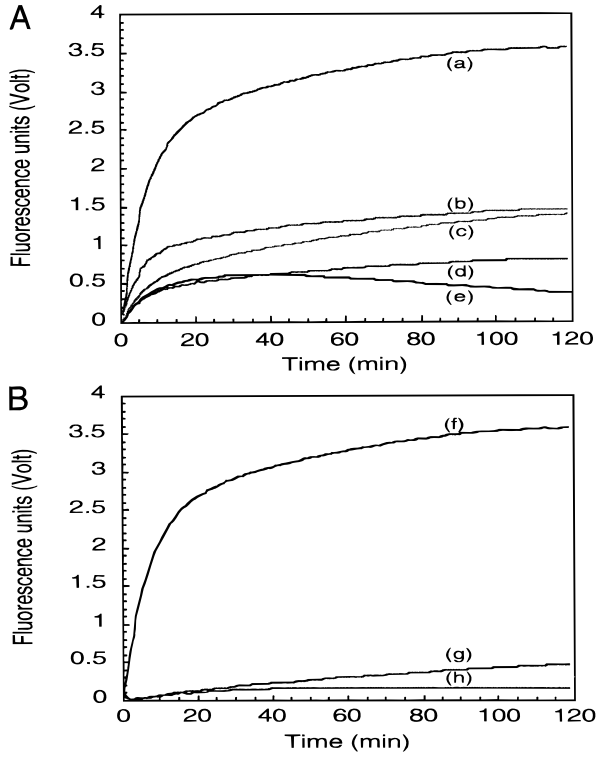

FIGURE 7: Fluorescence assay of the reassociation of dissociated tubulin subunits into active dimers. For a detailed explanation of the rationale and interpretation of the experiments shown in this figure, see the Results. (A) Reconstitution of colchicine binding activity in tubulin solutions treated with 0.2 M Tris-HCl buffer at pH 8.0. (a) Control plot. Tubulin (80 μM in PME buffer) was diluted 40-fold in Tris buffer at pH 8.0 supplemented with a reconstitution mixture as described in the Results. The tubulin solution (2 μM) was incubated on ice for 30 min and subsequently transferred in a spectrofluorimeter. Then, colchicine (50 μM) was added, and fluorescence measurements of colchicine binding over time were recorded as described in Materials and Methods. (b) Same as in (a), except that the tubulin concentration was 0.8 μM. (c) Reconstitution experiment. Tubulin (80 μM in PME buffer) was diluted in 0.2 M Tris-HCl buffer at pH 8.0 to a final protein concentration of 2.2 μM and the mixture incubated for 30 min on ice. The solution was transferred in a spectrofluorimeter. Then, colchicine (50 μM) was added together with a reconstitution mixture, and the fluorescence vs time plot was recorded. The final tubulin concentration was 2 μM. (d) Same as in (a), except that the tubulin concentration was 0.4 μM. (e) Tubulin (80 μM in PME buffer) was diluted in 0.2 M Tris-HCl buffer at pH 8.0 to a final protein concentration of 2 μM and the mixture incubated for 30 min on ice. The solution was transferred in a spectrofluorimeter. Then, colchicine (50 μM) was added, and the fluorescence vs time plot was recorded. (B) Reconstitution of colchicine binding activity in tubulin solutions treated with 0.2 M Tris-HCl buffer at pH 8.5. (f) Control plot. Tubulin (80 μM in PME buffer) was diluted 40-fold in 0.2 M Tris-HCl buffer at pH 8.5 supplemented with a reconstitution mixture as described in the Results. The tubulin solution (2 μM) was incubated on ice for 30 min and subsequently transferred in a spectrofluorimeter. Then, colchicine (50 μM) was added, and fluorescence measurements of colchicine binding vs time were recorded as described in Materials and Methods. (g) Reconstitution experiment. Tubulin (80 μM in PME buffer) was diluted in 0.2 M Tris-HCl buffer at pH 8.5 to a final protein concentration of 2.2 μM and the mixture incubated for 30 min on ice. The solution was transferred in a spectrofluorimeter. Then, colchicine (50 μM) was added together with a reconstitution mixture, and the fluorescence vs time plot was recorded. The final tubulin concentration was 2 μM. (h) Tubulin (80 μM in PME buffer) was diluted in 0.2 M Tris-HCl buffer at pH 8.5 to a final protein concentration of 2 μM and incubated for 30 min on ice. The solution was transferred in a spectrofluorimeter. Then, colchicine (50 μM) was added, and the fluorescence vs time plot was recorded.

about 12−15% of the initially dissociated tubulin subunits could reassociate into functional dimers, as seen from the plateau reached by curve g in Figure 7B (Table 1).

Several control experiments were carried out to ensure that the origin of the fluorescence change was indeed the binding of colchicine to tubulin $\alpha\beta$-dimers that had reassociated into a functional state in the neutral mixture of Tris and Pipes buffers. We verified that separated tubulin subunits were devoid of colchicine binding activity in the reconstitution buffer. The α- and β-subunits were separated by affinity chromatography as described above (Figure 4), and their individual ability to bind colchicine was assayed in the reassociation mixture of neutral Tris and Pipes buffers instead of neutral Tris buffer. The same inability to bind colchicine was found for the isolated α- or β-subunits in the Tris/Pipes mixture as in neutral Tris buffer (see above, Figure 4A). We also verified that the undissociated tubulin dimers present at the end of the Tris incubation step did not participate in the increase of colchicine binding activity observed during reassociation experiments; after a 30 min incubation in dissociating Tris buffer, undissociated tubulin dimers (and free α-tubulin subunits) were absorbed onto YL$^1/_2$ affinity gel, using batchwise incubations. Suspensions were then either assayed immediatly for colchicine binding activity or placed for various amounts of time in the reassociation mixture prior to assay of colchicine binding activity. The results showed a complete absence of detectable changes in colchicine binding activity upon incubation of undissociated dimers (and isolated α-subunits) in the reconstitution buffer (not shown). Other control experiments were run to eliminate the possibility of artifactual variations of fluorescence. In the reassociation mixture of Pipes and Tris buffers, the fluorescence of native tubulin or colchicine alone remained constant for 2 h (not shown). When colchicine binding was assayed in alkaline Tris buffer instead of in the neutral Tris/Pipes mixture, fluorescence plots did not show significant reassociation. In Tris buffer at pH 8.0 (curve e in Figure 7A), the initial slope of the fluorescence plot was comparable to that obtained in the reconstitution buffer, consistent with the equality of the initial concentration of active dimers under both conditions. The fluorescence subsequently reached a maximum and then decreased. Such a decrease indicated slow dissociation of the initially formed tubulin−colchicine complexes as expected from previous experiments (Figure 6A). In Tris at pH 8.5, fluorescence changes could barely be detected during the whole course of the experiment (curve h in Figure 7B).

DISCUSSION

This study shows that tubulin dimers can be dissociated into α- and β-tubulin subunits and that the subunits can be separated under nondenaturing conditions in Tris-based buffers. The physical isolation of separated α- and β-tubulin subunits was achieved using mAb YL$^1/_2$ which specifically recognizes tyrosinated α-subunits. There have already been several reports on the dissociation of tubulin, and in most cases, dissociation was described in Pipes buffer (*28*−*34*). Therefore, we were surprised that our immunoaffinity assay with mAb YL$^1/_2$ could not detect any dissociation of tubulin in Pipes, whether in batch solution or by column chromatography. Some dissociation should have occurred on the basis of the tubulin concentrations used (0.5 and 2 μM) and

the reported equilibrium dissociation constant ($10^{-7}-10^{-6}$ M) (*28*−*34*). Several possibilities might explain this discrepancy. First, if the actual value of K_d were 10^{-7} M or lower, batch assays would not be sensitive enough to measure the small extent of dissociation at 2 μM tubulin. Also, the failure to monitor any dissociation by chromatography could be due to the fact that the concentration of tubulin in the immunoabsorbent gel was much larger than K_d. Under such conditions, dissociated β-tubulin subunits would have been continuously re-bound by free α-subunits and thus strongly retained on the column (*48*). Another possibility is that the tyrosination of the α-subunit decreases the value of K_d in Pipes buffer. Because we have exploited the specificity of the mAb YL$^1/_2$ for tyrosinated α-tubulin to follow the dissociation of tubulin into its α- and β-subunits, we had to use homogeneous tyrosinated tubulin. Some other studies were performed with heterogeneous tubulin and could have detected only the dissociation of the nontyrosinated fraction that had a higher value of K_d. It could also be that we have actually measured the dissociation of αβ-tubulin after binding to the mAb YL$^1/_2$−Sepharose 4B gel, and it also possible that the interaction between the α- and β-subunits is stabilized by the binding of the mAb YL$^1/_2$ to the tyrosinated α-subunit. Independent of any explanation, a practical consequence of our results is that Pipes buffer seems to be inappropriate for isolation of the large amounts of tubulin subunits needed for biochemical studies.

One question that arises concerns the reason Tris-based buffers are more efficient than other buffers in separating tubulin subunits. The stability of the interactions between proteins is markedly influenced by the composition of the solvent. Neutral salts and uncharged solvent additives have been classified as "stabilizers" [e.g., $(NH_4)SO_4$, phosphate, glycerol, sucrose, etc.] or "destabilizers" (e.g., SCN^-, guanidinium, Li^+, urea, etc.) of protein structure (*49*). The difference between Pipes and Tris buffers on the stability of the tubulin αβ-heterodimer is probably due to the relative destabilizing effect of an amine buffer compared to phosphate or sulfonate buffer (*50*). In addition to this marked influence of the buffer, we have also found that the tubulin dissociation was increased at alkaline pH. Raising the pH from 7.0 to 8.5 greatly increased the rate and/or extent of dissociation. Therefore, deprotonation of some basic groups resulted in weakening of the interface between the α- and β-subunits, either by abolishing electrostatic interactions in the contact area or by increasing overall electrostatic repulsion.

A most important aspect of Tris action on the tubulin dimer was the ability to dissociate the dimers in the absence of detectable denaturation. Tubulin subunits could be separated in Tris at pH 7.0, under conditions in which nondissociated tubulin dimers apparently retained full functionality. Preformed tubulin−colchicine complexes survived for a long period of time in dissociating buffers. Although dissociation of the α- and β-tubulin subunits completely abolished colchicine binding, circular dichroism spectroscopy showed that the separated subunits retained a large fraction of their native conformation. Finally, and most importantly, dissociated tubulin subunits could regain colchicine binding activity upon reassociation under proper buffer conditions. Under our reconstitution conditions, about 25% of the initially dissociated tubulin subunits reassociated into active complexes. This value yields an estimate of the minimal proportion of reassociation-competent tubulin subunits present in Tris-dissociated tubulin solutions. Factors other than tubulin denaturation are most probably limiting the yield of tubulin subunit reassociation. Tubulin has been designed by evolution to cycle between a soluble and a polymerized state, and this function as a self-associating protein could explain the strong tendency of tubulin to aggregate. Dissociated α- and β-subunits have shown the same tendency toward aggregation, and the opportunity of dissociated α- and β-subunits to either aggregate or reassociate into the native heterodimer probably has been an important limiting factor in reconstitution experiments. A systematic search for ideal conditions that minimize aggregation and optimize reassociation should increase the yield of tubulin reconstitution. Furthermore, even under the most pessimistic hypothesis that only 25% of separated tubulin subunits have a conserved structure, comparative assays of separated subunit functions (nucleotide binding, drug binding, interaction with motors or MAPs, etc.) remain possible.

This study provides an example of the potential of the Tris-based tubulin dissociation method in dissecting molecular interactions, in the case of the tubulin−colchicine interaction. Consistent with previous reports, we find that the binding of colchicine to tubulin stabilizes the tubulin dimeric state and protects tubulin against dissociation (*29*, *30*, *33*, *34*, *45*−*47*). In native tubulin, the main binding site for colchicine is carried by the β-subunit, but some binding to α-tubulin also occurs (*51*−*54*). The stability of tubulin−colchicine complexes could therefore indicate that the colchicine binding site is located at the subunit interface so that binding the drug would cross-link the αβ-dimer. Alternatively, the interactions between the α- and β-subunits could have been strengthened by the conformational changes known to occur in tubulin upon colchicine binding (*55*−*57*). A long-standing question concerning the mechanism of colchicine interaction with tubulin is whether colchicine interaction with tubulin subunits requires an intact dimer structure. We find that solutions of separated tubulin subunits are unable to bind colchicine in a reconstitution buffer while equimolar mixtures of tubulin subunits have colchicine binding activity in the same buffer. These results provide strong evidence that colchicine binding requires not only both subunits but also the proper quaternary interactions between them. In apparent contrast with these data, it has been reported that colchicine could bind to isolated tubulin β-subunits after dissociation of tubulin in the presence of lactoperoxidase (*35*). However, lactoperoxidase forms a tight complex with the dissociated α- or β-subunit and could "mimic" either tubulin subunit. If so, the formation of a β-tubulin−lactoperoxidase complex could induce a conformational change in the β-subunit that restores colchicine binding. Lactoperoxidase not only would have an affinity high enough to displace the α-subunit and dissociate tubulin but also would "replace" the domain of α-tubulin that normally interacts with β-tubulin.

In conclusion, this study describes conditions which allow the quantitative dissociation of tubulin into "native" α- and β-subunits that can be separately isolated. The availability of the separated subunits should facilitate analysis of the interactions of the individual α- and β-subunits with ligands such as MAPs, molecular motors, nucleotides, drugs, or cell organelles. The separation of tubulin subunits under non-

denaturing conditions may also contribute to the understanding of the mechanism of tubulin dimer formation. In vivo, it is known that the formation of tubulin dimers from isolated subunits is a complex process mediated by a series of chaperones (*58*). The availability of separated native tubulin subunits could help in the deciphering of the biochemical mechanisms involved in the interaction of tubulin with chaperones.

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