Cold Depolymerization of Microtubules to Double Rings: Geometric Stabilization of Assemblies†

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ABSTRACT: The kinetic pathway of microtubule depolymerization at 0 °C has been examined. Microtubules made of MAP-containing and MAP-free tubulins were depolymerized at 0 °C in the presence of [^{3}H]GDP or [^{3}H]GTP or of trace amounts of ^{125}I dimeric tubulin. The products of depolymerization were separated on a column, their structures were identified by electron microscopy, and the time course of incorporation of ^{3}H or ^{125}I labels in the different components of the system was determined. Two predominant assembly states of tubulin found in the nonmicrotubule state were α–β dimers and double rings. Kinetic data indicate that ring formation from disassembling microtubules does not occur by direct coiling of protofilaments as previously thought, but disassembling GDP subunits are in very rapid equilibrium with curved oligomers that are kinetic intermediates in the isodesmic assembly of GDP–tubulin. The formation of oligomers and rings from dimers, at concentrations as low as 10 μM, is much faster than nucleotide exchange on α–β-tubulin. Disassembly of double rings, in contrast, is slower than nucleotide exchange on α–β-tubulin, by 1 order of magnitude in the absence of MAPs and 2 orders of magnitude in the presence of MAPs. These results support the model proposed previously to explain spontaneous oscillations in microtubule assembly. They are consistent with the existence of an equilibrium between two conformations of tubulin, "straight", i.e., microtubule forming, and "curved", i.e., ring forming, under the allosteric control of bound nucleotide. The straight conformation requires the presence of two ionizable hydroxyls on the γ-phosphate in GTP or GDP–P$_i$.

The pathway of the microtubule assembly–disassembly cycle has been the subject of numerous studies. A crucial point has been whether the 42S double rings[1] frequently seen in tubulin systems were actual intermediates in the polymerization process of microtubules. Although such a possibility was evoked early (Kirschner, 1978; Scheele & Borisy, 1979), this hypothesis was obviated by the statistical thermodynamic analysis of microtubule nucleation and growth of Erickson and Pantaloni (1981). Further structural and biochemical studies have shown that rings depolymerize prior to microtubule assembly into small oligomers which are precursors of the nuclei (Weisenberg et al., 1976; Mandelkow et al., 1980; Pantaloni et al., 1981; Bordas et al., 1983; Spann et al., 1987). In the presence of microtubule-associated proteins (MAPs),[2] oligomers consisting of tubulin and MAPs also form the elongating blocks in microtubule assembly (Carlier et al., 1984a,b). On the microtubule depolymerization side of the cycle, however, it is not clear whether the observed rings are formed directly from the MAP-containing microtubules or from α–β-tubulin dimers that would first dissociate from microtubules and then assemble into rings. Electron microscopic observations showing curling ends of depolymerizing microtubules after cooling below 10 °C (Erickson, 1974; Kirschner et al., 1974; Bordas et al., 1983; Mandelkow & Mandelkow, 1985) seemed to favor the first pathway, but recent more refined cryomicroscopy studies have pointed out the possibility of artifacts leading to such images (Wade et al., 1989). Furthermore, EM studies alone cannot establish a given pathway. Recently, Howard and Timasheff (1986) have examined the dependence of the MAP-free α–β-tubulin $\rightleftharpoons$ double-ring equilibrium on the state of phosphorylation of the guanine nucleotide bound at the exchangeable E site of tubulin. Their finding that GDP–tubulin has a greater propensity to form rings than GTP–tubulin led these authors to suggest that, in the cold disassembly of microtubules, rings formed of GDP subunits could be generated directly from protofilaments of GDP microtubules without prior dissociation into dimers. Experiments were, therefore, undertaken to test this hypothesis. The kinetic pathway from microtubules to 42S rings at 0 °C has been investigated by using a combination of ^{3}H-nucleotide and ^{125}I-tubulin labeling at different times following depolymerization during the establishment of the 6S $\rightleftharpoons$ 42S equilibrium, and the kinetics of labeling of the different species

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[1] The value of 42 ± 1 S for the sedimentation coefficient of the double rings has been obtained by rigorous analysis of the ultracentrifuge data in terms of the proper transport theory (Frigon & Timasheff, 1975a). Other reported values seem to reflect neglect either of the pressure erosion of hypersharp peaks or of the fact that the ultracentrifuge patterns are those of reaction boundaries. By electron microscopy, all the observed double rings appear to have the same morphology (Kirschner et al., 1974; Erickson, 1974; Frigon & Timasheff, 1975a; Bordas et al., 1983), as pointed out by Vallee and Borisy (1978), who have attributed the variation of the *s* values to the presence or absence of MAPs.

[2] Abbreviations: GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GMPPCP; guanosine 5′-(β,γ-methylenetriphosphate); GMPPNP, guanosine 5′-(β,γ-imidotriphosphate); GMPCPP, guanosine 5′-(α,β-methylenetriphosphate); MAP, microtubule-associated protein; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EM, electron microscope.

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involved have been determined. The results of these experiments led to a view of the depolymerization process which differs from the currently favored one, and yield rough quantitative estimates of the rates of the rapid isodesmic polymerization of tubulin into oligomers and rings, the nucleotide exchange on tubulin, the opening of rings, and the dissociation of MAPs from rings.

Materials and Methods

Preparation of Microtubule Protein and Pure Tubulin. Porcine brain tubulin was prepared by two cycles of polymerization–depolymerization (Shelanski et al., 1973). The microtubule pellet obtained at the end of the second cycle was homogenized in buffer G (0.1 M MES, pH 6.8, 1 mM EGTA, 0.5 mM EDTA, 0.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 8 M glycerol, and 200 μM GTP),[2] adjusted to a concentration of 5–10 mg/mL, and stored at −20 °C.

Pure tubulin was prepared by chromatography on phosphocellulose P11 (Whatman) (Weingarten et al., 1975), following a third cycle of polymerization of microtubule proteins. The eluted tubulin was concentrated by ultrafiltration, adjusted to 0.05 M MES, pH 6.8, 0.25 mM $MgCl_2$, 0.5 mM EGTA, 4 M glycerol, and 0.1 mM GTP, and stored at −80 °C at a concentration of 5–10 mg/mL.

Preparation of ^{125}I-Labeled Phosphocellulose–Tubulin. Iodinated PC–tubulin was prepared by using the Bolton–Hunter reagent, as described by Carlier et al. (1980). It was purified further by one cycle of polymerization–depolymerization and stored in 0.05 M MES, pH 6.8, 0.5 mM EGTA, 0.25 mM $MgCl_2$, 4 M glycerol, and 200 μM GTP at −80 °C at a concentration of 0.8 mg/mL.

Pure ^{125}I-tubulin, once chromatographed on Bio-Gel A 1.5 m at the tracer concentration (0.4 μM) used in the experiments, yielded a major peak of radioactivity (90%) at the elution position of dimeric tubulin. A small portion (10%) of the radioactive material was found in the void volume. All elution patterns were corrected for this (presumably aggregated) ^{125}I-tubulin which contaminated the ring fraction.

Preparation of Dimeric GDP–Tubulin. Phosphocellulose tubulin was polymerized in buffer C (50 mM MES, pH 6.8, 0.5 mM EGTA, 0.25 mM $MgCl_2$, and 4 M glycerol) supplemented with 0.5 mM GTP and 6 mM $MgCl_2$. Microtubules were pelleted at 130000g, 37 °C, for 50 min and then suspended in ice-cold buffer B (0.1 M MES, pH 6.8, 1 mM EGTA, and 0.5 mM $MgCl_2$). After 30 min of depolymerization at 0 °C, the solution was clarified by centrifugation at 50000g, 4 °C, for 40 min. The GDP–tubulin concentration in the supernatant was usually equal to 20–25 μM.

Separation and Characterization of Microtubule Depolymerization Products. (1) In the Presence of MAPs. Microtubule protein was polymerized in buffer A (0.1 M MES, pH 6.8, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.5 mM GTP, and 4 M glycerol). Microtubules were sedimented for 90 min at 160000g through a 30% sucrose cushion (2 mL) in buffer A without GTP to prevent contamination of the pellet by free nucleotides. Microtubules were then depolymerized at 0 °C by rapid homogenization in a Kontes Teflon–glass homogenizer (3 strokes) in ice-cold buffer B containing either [^{3}H]GDP or [^{3}H]GTP. In the experiments where the dimeric tubulin was labeled, the nucleotide was unlabeled in buffer B, which contained 0.4 μM ^{125}I-tubulin. In some experiments, microtubules were depolymerized in buffer B in the absence of any labeled molecule, the labeled compound was then added at defined times following the onset of depolymerization, and the time course of incorporation of the label into the different fractions was followed, as will be described below.

Time zero of the experiment was taken as the time of the first stroke of the pestle in the homogenizer. Complete depolymerization required 20–30 s. At different time intervals following the addition of the tracer compound, 0.85-mL aliquots of the protein solution (2–5 mg/mL) were processed for fractionation by gel filtration (Erickson, 1974; Pantaloni et al., 1981) on a 0.9 × 43 cm column of Bio-Gel A 1.5 m (Bio-Rad), topped with a 10-cm layer of Sephadex G 25 fine (Pharmacia) that separated protein from free nucleotide in 2 min as verified with a mixture of Blue Dextran and Phenol Red. The column was equilibrated in buffer B at 4 °C. The flow rate was set at 0.2 mL/min, which ensured an efficient separation of oligomers and dimeric tubulin in less than 10 min.

Tubulin concentration was determined spectrophotometrically by using an extinction coefficient of 1.2 mg^{-1} cm^2 (Detrich & Williams, 1978). The concentrations of ^{3}H-nucleotide and ^{125}I-tubulin in the fractions were determined with a Packard CA2000 scintillation counter.

(2) In the Absence of MAPs. In absence of MAPs, at high $MgCl_2$ concentration, the ring $\rightleftharpoons$ tubulin equilibrium is faster than when MAPs are present, the rate of reequilibrium being rapid relative to the time of a gel filtration experiment (Frigon & Timasheff, 1975a,b; Howard & Timasheff, 1986). Since this rendered impossible the separation of oligomers by gel filtration, the identification of the microtubule depolymerization products was carried out after cross-linking them with glutaraldehyde. Phosphocellulose–tubulin was polymerized into microtubules at 37 °C in buffer C supplemented with 0.5 mM GTP and 6 mM $MgCl_2$. The microtubule solution (6 mL) was centrifuged for 90 min at 37 °C at 160000g through a 2-mL cushion of 30% sucrose in 0.05 M MES, pH 6.8, 0.5 mM EGTA, 6 mM $MgCl_2$, and 4 M glycerol. The microtubule pellet was homogenized at 0 °C in a 0.1 M MES, pH 6.8, 1 mM EGTA, and 12 mM $MgCl_2$ buffer containing 0.2 mM [^{3}H]GDP or 0.4 μM ^{125}I-PC–tubulin and unlabeled GDP. At various times following homogenization (20 s to 5 min), an aliquot of the solution was removed and fixed for 1 min with 0.2% (20 mM) glutaraldehyde at 0 °C. Cross-linking was stopped by addition of 40 mM NH_2OH, and the sample was loaded immediately on a Bio-Gel A 1.5 m–Sephadex G 25 column, following the same procedure as in the presence of MAPs. Controls made by electron microscopy showed that no microtubules remained 20 s after the beginning of homogenization.

Analysis of Chromatographic Data. The levels of labeling of the excluded and included components were calculated on one to three fractions of the respective peaks. It must be pointed out that in the study of the products of depolymerization of MAP-rich microtubules some dissociation of rings into dimers may have occurred during the course of the chromatography. Therefore, the trailing fractions of the excluded material may be heterogeneous. Since MAPs, however, slow down the dissociation of rings, any dimers present in the trailing end of the excluded fraction must originate from the rings deposited initially on the column. Their specific degree of labelling was, therefore, the same as that of initial rings, as can be verified on Figure 4B. In the studies with pure tubulin, the excluded fraction contained only cross-linked material which cannot undergo rapid association–dissociation.

Electron Microscopy. The morphology of the oligomers was examined by electron microscopy in a Philips EM 410 instrument, using negative staining by 1% uranyl acetate.

Kinetics of Assembly of Dimeric Tubulin into Oligomers in the Presence of GTP and GDP. The polymerization of 6S

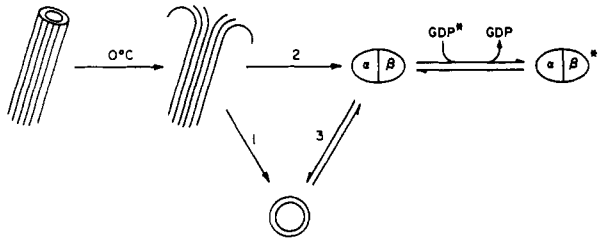

FIGURE 1: Proposed pathways of the cold depolymerization of microtubules. The star on the α–β subunit means that labeled GDP is bound to it.

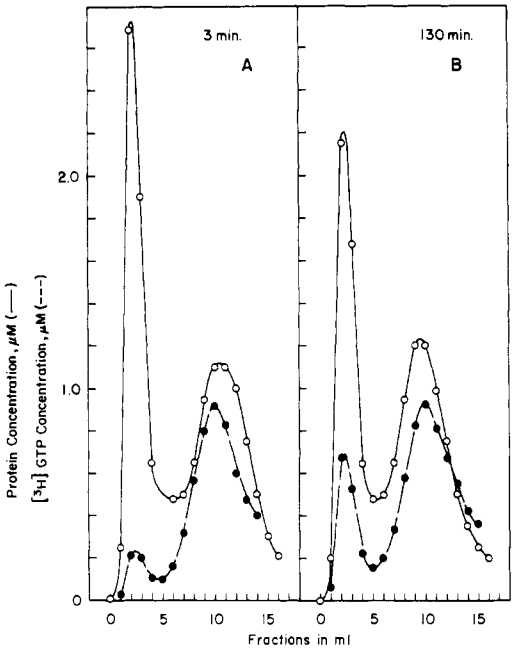

FIGURE 2: Incorporation of [^{3}H]GDP into the ring and dimeric α–β-tubulin fractions as a function of time following the cold depolymerization of MAP-rich microtubules in buffer B containing 200 μM [^{3}H]GDP. The elution pattern from the Bio-Gel A 1.5 m column is shown. Open circles and solid lines, protein concentration; closed circles and dashed lines, ^{3}H radioactivity incorporated into the tubulin. The time corresponds to time elapsed after the start of microtubule depolymerization. The flow rate was 0.2 mL/min.

tubulin into oligomers and 42S rings was monitored at 10 °C by light scattering. The increase in the intensity of light scattered at 90° from the incident beam was measured at 350 nm using a Spex Fluorolog 2 spectrofluorimeter, following the simultaneous addition of 12 mM $MgCl_2$ and 0.5 mM either GDP or GTP to a 20 μM solution of dimeric GDP–tubulin in buffer B.

Results

A sketch of the working model used at the outset of this study is presented in Figure 1. Two simple hypothetical pathways are presented for microtubule depolymerization into rings and dimers: either rings emerge directly from microtubules by the coiling of protofilaments (pathway 1) or microtubules first depolymerize into dimeric tubulin (pathway 2) which then self-assembles into double rings by equilibrium 3. The nucleotide on tubulin dimer can exchange with free nucleotide in solution.

In order to quantify the contribution of these processes to the overall real reaction, the time course of incorporation of ^{3}H-nucleotide or ^{125}I-tubulin was monitored both *during* ring formation and *long after the completion of depolymerization*, when equilibrium between rings and dimers had been reached. These studies were performed on both MAP-rich and MAP-free microtubules.

Depolymerization of MAP-Rich Microtubules. When a pellet of 3 times cycled microtubule protein was depolymerized at 0 °C in a buffer containing [^{3}H]GDP or [^{3}H]GTP, and the products of depolymerization were fractionated on Bio-Gel A 1.5 m at different times between 3 min and 2 h following homogenization, the labeled nucleotide was incorporated slowly into the oligomer fraction, but much more rapidly into dimeric tubulin. Figure 2 shows that 3 min after the onset of depolymerization, free tubulin had exchanged 82% of its E-site nucleotide. This value is in agreement with the known rate constant (0.15 s^{-1}) for GDP dissociation from tubulin. In contrast, the oligomer fraction had exchanged only 7.4%. After 2 h of incubation, the oligomer fraction was ^{3}H-labeled to 32%. This last piece of data is consistent with the previously reported value of 120 min for the half-time of tubulin–double-ring exchange in the presence of MAPs (Pantaloni et al., 1981). Using double-labeled [γ-^{32}P]- and [^{3}H]GTP in the above experiment yielded superimposable distributions of the two labels, which confirmed that no GTP hydrolysis was accompanying nucleotide exchange on either dimers or oligomers. When MAP-containing microtubules were depolymerized in the presence of ^{125}I-tubulin and immediately processed for gel filtration, the oligomer fraction was found to be highly labeled. Figure 3A shows that the excluded oligomer fraction, which

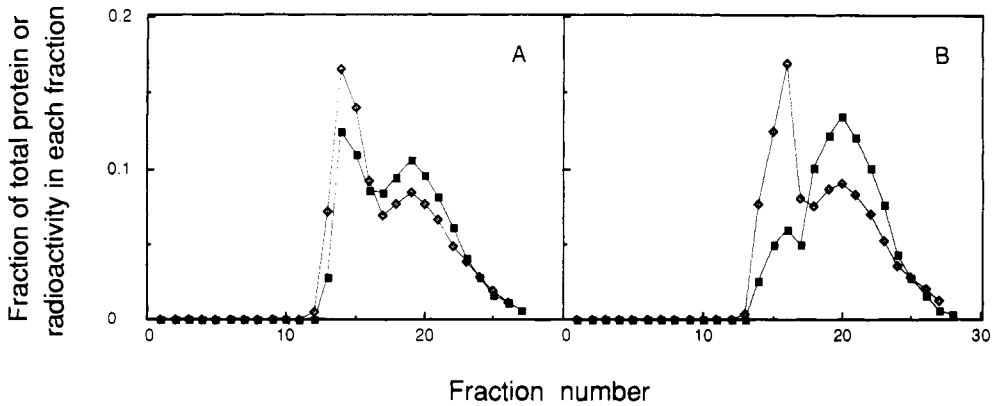

FIGURE 3: Comparison of the rapid initial incorporation of ^{125}I-tubulin into rings in the early stages of microtubule depolymerization and after attainment of the ring $\rightleftharpoons$ α–β-tubulin dimer equilibrium. (Panel A) MAP-rich microtubules were depolymerized (80–100 μM tubulin in cold buffer B containing 0.4 μM ^{125}I-tubulin) and immediately loaded (t = 20 s) on the Bio-Gel column. (Panel B) Parallel experiment in which microtubules were depolymerized in buffer B; the solution was kept on ice for 10 min, at which time 0.4 μM ^{125}I-tubulin was added to the sample, which was loaded on the Bio-Gel column 20 s later. The open symbols represent protein concentration, the closed symbols ^{125}I radioactivity. The total areas under the protein and radioactivity elution patterns were normalized to 1. The ordinate represents the fraction of total protein or radioactivity contained in each fraction.

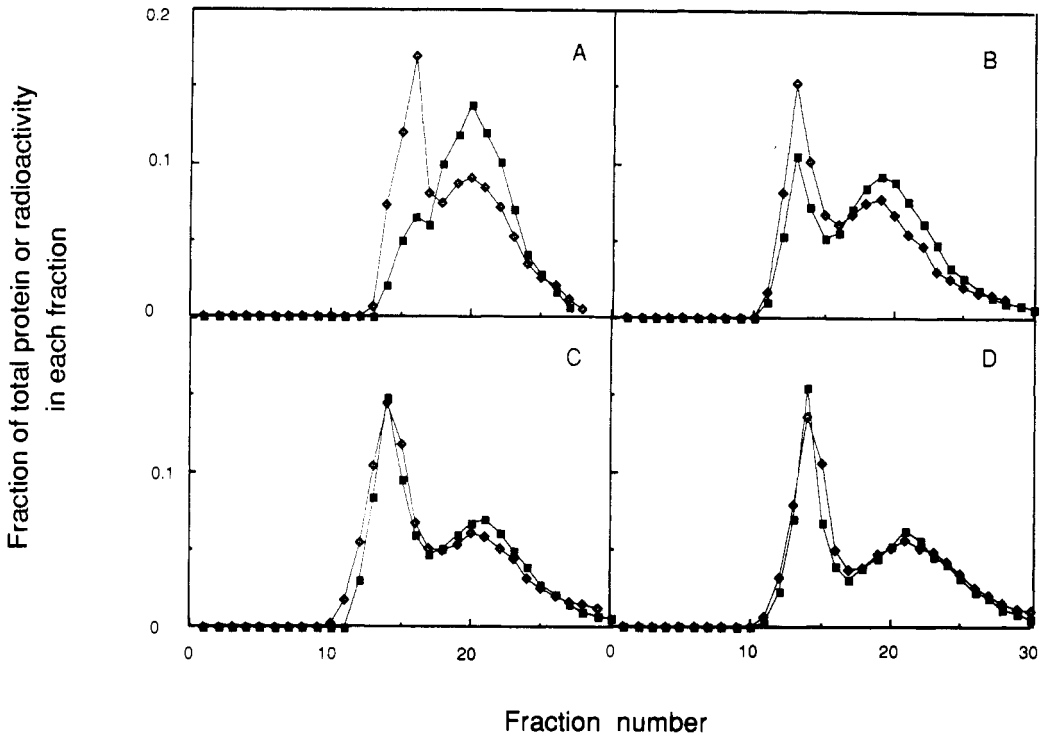

FIGURE 4: Time course of ^{125}I-tubulin incorporation into rings at equilibrium. MAP-rich microtubules were depolymerized in buffer B at 0 °C. The solution was kept on ice for 10 min before addition of 0.4 μM ^{125}I-tubulin. Aliquots were processed for the separation of rings and dimers at different time intervals following the addition of ^{125}I-tubulin. (A) 10 s; (B) 15 min; (C) 30 min; (D) 120 min. The open and closed symbols represent protein concentration and ^{125}I radioactivity, respectively. The data are normalized as described in Figure 3.

comprised approximately 60% of the total protein, also contained about 40–45% of the total amount of ^{125}I-tubulin present at time zero of the depolymerization process. If the sample was chromatographed 20 min following depolymerization, 50–55% of the label was incorporated into the ring and oligomers fraction, which was the maximum extent of labeling reached.

On the other hand, if the microtubules were depolymerized at 0 °C, and ^{125}I-tubulin was added 10 min after the onset of depolymerization, a weak but measurable labeling of the excluded fraction was observed in the elution pattern of the sample chromatographed immediately after addition of the label (Figure 3B). Labeling of the excluded fraction increased then slowly with time and reached the same level as in the preceding experiment, in which ^{125}I-tubulin had been present from time zero in the depolymerization buffer. The evolution of the labeling is shown in Figures 4 and 5. Strictly identical slow kinetics of incorporation were obtained whether ^{125}I-tubulin was added 5, 10, or 45 min after the onset of depolymerization. In all cases, the incorporation of labeled tubulin into the oligomers at equilibrium could not be described by a single kinetic step, as shown in Figure 5. The simplest kinetic scheme that can be proposed, given the moderate degree of temporal resolution of the chromatographic method used, involves two steps. A rapid phase accounted for the incorporation of ca. 20% of the total label into the oligomer fraction and a slower phase for about 30%, so that, at infinite time ($\sim$1 h), dimers and oligomers were equally labeled (50% label in each). The half-time for the slow phase was 15–20 min. In summary, the oligomers became highly labeled rapidly when depolymerization was carried out in the presence of labeled tubulin, while, at equilibrium, only a small fraction of the oligomers was labeled rapidly, the bulk of the labeling occurring by a slow process.

Comparison of the highly contrasting results obtained with ^{3}H-nucleotide and ^{125}I-tubulin leads to the conclusion that,

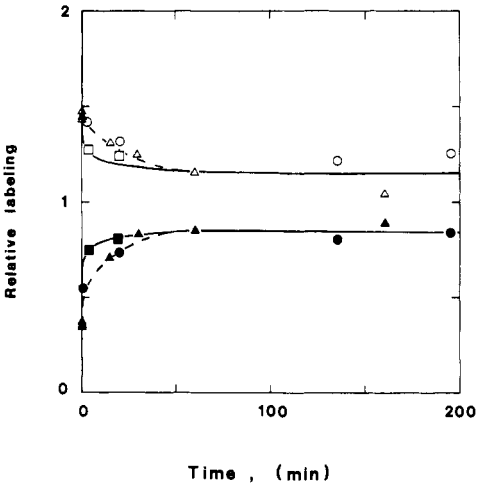

FIGURE 5: Time course of the ^{125}I radioactive labeling of rings and dimeric tubulin generated by the cold depolymerization of MAP-rich microtubules. Microtubule depolymerization was carried out either in buffer B containing ^{125}I-tubulin (■, □) or in buffer B without ^{125}I-labeled tubulin. In the latter case, ^{125}I-tubulin was added 10 min (▲, △) or 2 h (●, ○) after microtubule depolymerization. One-milliliter aliquots of the microtubule depolymerization products at 80–100 μM were then loaded on the separating column at the indicated times following the addition of labeled tubulin. Open symbols, labeling of dimeric tubulin; closed symbols, labeling of rings. The ordinate scale is defined by normalizing to 1 the identical relative labelings of rings and dimeric tubulin at infinite time.

during microtubule depolymerization, association of dimers into oligomers occurs at a rate faster than nucleotide exchange on dimeric tubulin. The results also indicate that the equilibrium between rings and dimers is established in less than 5 min after the depolymerization of microtubules.

Depolymerization of MAP-Free Microtubules. The depolymerization of MAP-free microtubules was performed in a buffer containing 200 μM [^{3}H]GDP and 12 mM $MgCl_2$, since

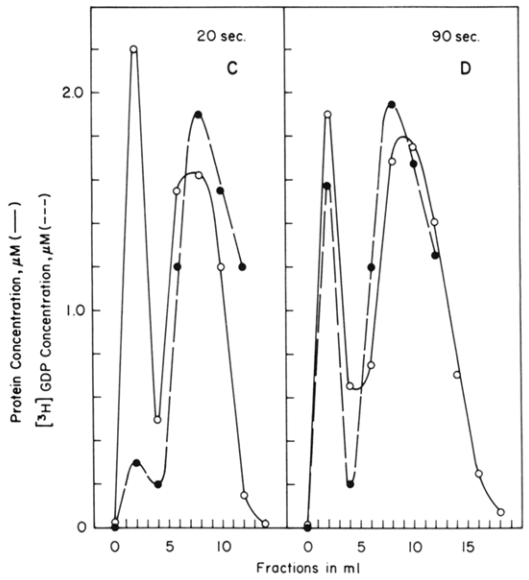

FIGURE 6: Time course of the incorporation of [^{3}H]GDP into rings during the cold depolymerization of MAP-free microtubules. MAP-free microtubules (PC–tubulin) were depolymerized in buffer C containing 200 μM [^{3}H]GDP, fixed with glutaraldehyde for 1 min at the times indicated, and loaded on the Bio-Gel column for separation of cross-linked rings and tubulin dimers. Open circles, solid line, protein concentration; closed circles, dashed lines, ^{3}H radioactivity.

the tubulin ⇌ double-ring equilibrium is favored toward self-assembly by GDP and Mg^{2+} ions (Howard & Timasheff, 1986). The depolymerization products were frozen in the state of aggregation which exists at different times following depolymerization by rapid fixation with glutaraldehyde.[3] The resolution on the gel filtration column of the glutaraldehyde-fixed depolymerization mixture at 20 and 90 s after depolymerization is shown in panels A and B, respectively, of Figure 6. The two-peak patterns obtained were similar to those found in the presence of MAPs. The morphology of the oligomers constituting the first fraction was examined by electron microscopy. A typical picture, shown in Figure 7, displays a number of assembled structures, the most prominent ones being double rings. Other structures encompass aggregates of rings, twisted rings, and partly uncoiled rings. No ordered structures having a curvature different from that found in rings were observed. Small oligomers appearing in the background seem structurally related to the intermediates in the double-ring ⇌ α–β-tubulin isodesmic equilibrium (Frigon & Timasheff, 1975b). Figure 6 shows that the labeling of dimeric tubulin with ^{3}H-nucleotide is more rapid than that of oligomers. For instance, 20 s following its dissociation from microtubules at 0 °C, dimeric tubulin had its E-site GDP 90% labeled, while the ring fraction was only 13.6% labeled. Full exchange of nucleotide in the ring fraction required >90 s. The time course of the increase in ring labeling is shown in Figure 8. The data indicate that equilibrium between rings and tubulin dimers is much faster than in the presence of MAPs. It is difficult to assess an accurate value for the exchange rate constant because samples at each time point were fixed by glutaraldehyde for 1 min before the chromatography. A reasonable value consistent with the data is $\sim 10^{-2}$ s^{-1}. When the depolymerization was performed in the presence of [^{3}H]GTP, the results obtained were essentially similar, but

[3] Although the cross-linking of rings and oligomers by glutaraldehyde may not be complete, the radioactive content of the cross-linked ring fraction that comes off the column gives a correct estimate of the extent of labeling of the rings at the time considered.

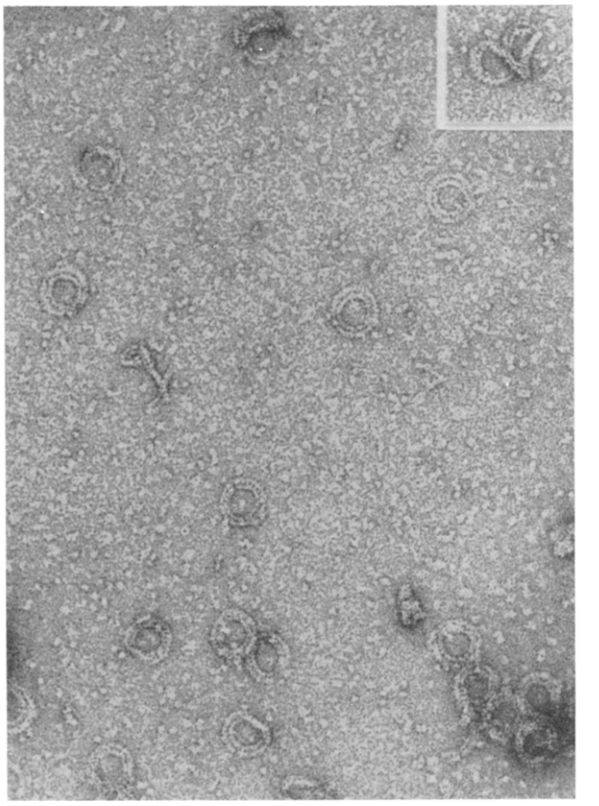

FIGURE 7: Identification of the cross-linked species present in the ring fraction following the cold depolymerization of MAP-free microtubules in buffer C. A sample of the ring fraction (0.8 mg/mL) was negatively stained for EM observation. Essentially, the only organized structures seen are double rings and open curved oligomers.

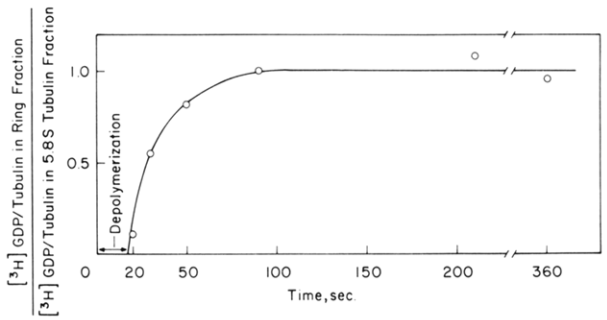

FIGURE 8: Time course of [^{3}H]GDP incorporation into rings following the cold depolymerization of MAP-free microtubules in buffer C containing 200 μM [^{3}H]GDP. Time zero is the time at which depolymerization by homogenization of the microtubule pellet was started. Each time point represents the time at which the 1-min cross-linking of the sample by 0.2% glutaraldehyde was initiated.

a kinetic analysis similar to that made for the depolymerization in the presence of GDP could not be carried out due to the progressively lower amounts of protein separated in ring form during approach to the tubulin ⇌ ring equilibrium, which is weaker in GTP than in GDP.

Depolymerization of microtubules made from pure tubulin in the presence of ^{125}I-tubulin, followed by fixation and chromatography as above, resulted in rapid total labeling of the ring fraction. Figure 9 shows that the oligomer fraction is practically as labeled as the dimer fraction as early as 20 s following microtubule depolymerization. An identical pattern was obtained if chromatography was carried out after 10 min

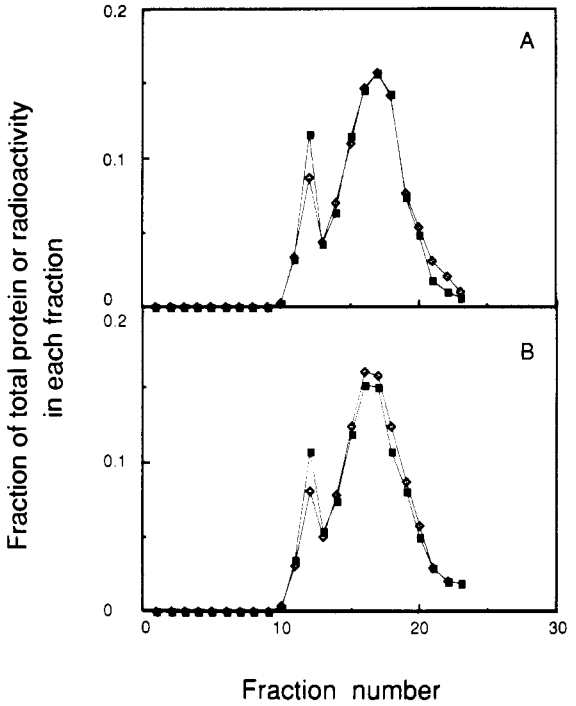

FIGURE 9: Incorporation of ^{125}I-tubulin into rings following the cold depolymerization of MAP-free microtubules in buffer C. Microtubules assembled from pure PC–tubulin were depolymerized at 0 °C in buffer C containing 0.4 μM ^{125}I-tubulin. Fixation by glutaraldehyde was started 20 s (panel A) and 10 min (panel B) after the onset of depolymerization. The chromatography was performed 1 min later. The open and closed symbols represent protein concentration and ^{125}I radioactivity. Data are normalized as in Figure 3.

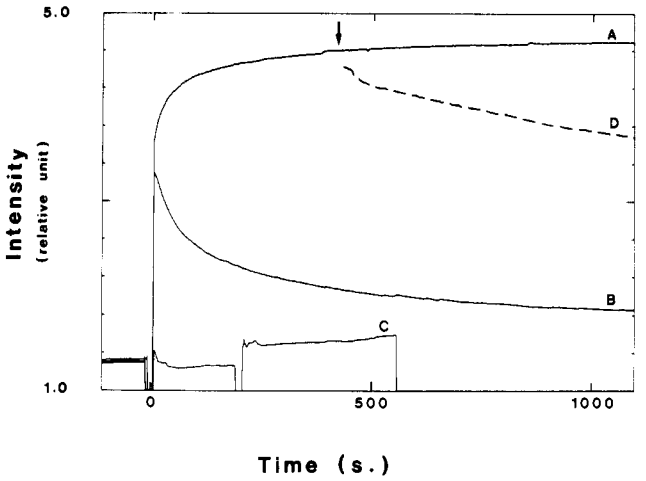

FIGURE 10: Light-scattering monitoring of the formation of rings and oligomers at 10 °C upon addition of magnesium ions to tubulin in the presence of GDP or GTP. GDP–tubulin (1:1 complex in buffer B) was prepared as described under Materials and Methods at a concentration of 20 μM and placed into the cell compartment of the Spex spectrofluorometer thermostated at 10 °C. At time zero, 12 mM $MgCl_2$ and either 500 μM GDP (curve A) or 500 μM GTP (curve B) were added simultaneously to the sample. Mixing was performed with a Teflon rod and required 3–5 s. In curve C, 500 μM GTP was added to GDP–tubulin at time zero, and 12 mM $MgCl_2$ 4 min later. Curve D (dashed line) shows the effect of the addition of 500 μM GTP (arrow) to oligomers of GDP–tubulin in 12 mM $MgCl_2$.

of incubation in the presence of ^{125}I-tubulin.

Isodesmic Self-Assembly of Dimeric Tubulin on Addition of Mg^{2+} Ions. The results of the preceding section seem to indicate that the assembly of dimeric tubulin into oligomers and rings is very fast, at any rate faster than nucleotide exchange on dimeric tubulin. In order to check this point, the kinetics of the isodesmic assembly were monitored by light scattering. A solution of GDP–tubulin dimer (20 μM, no free GDP) was prepared in buffer B (containing 0.5 mM Mg^{2+}) and placed in the thermostated compartment (10 °C) of the Spex fluorometer. Addition of 12 mM $MgCl_2$ at time zero to induce oligomer assembly resulted in a very fast (completed in 5 s) large increase in scattered light (Figure 10). This was followed by a slow increase of smaller amplitude over ~200 s (curve A). When 500 μM GTP was added to GDP–tubulin together with 12 mM $MgCl_2$, the same initial large burst in light scattering was again observed. Within a few seconds, it changed to a slow decrease, reaching a lower final plateau (curve B). The same scattering plateau was obtained by a monotone increase when 0.5 mM GTP was first added to GDP–tubulin, and 12 mM $MgCl_2$ 3.5 min later (curve C). Addition of GTP to GDP rings 6 min after the addition of $MgCl_2$ resulted in a slow decrease in light scattering (curve D). Although a detailed kinetic analysis would be risky because different oligomeric species scatter light differently, these data nevertheless show unambiguously that the assembly of dimeric tubulin into oligomers is much faster than nucleotide exchange on dimeric tubulin. The slow decrease in light scattering observed when Mg^{2+} and GTP are added simultaneously to GDP tubulin ($k \sim 10^{-2}$ s^{-1}) corresponds to the relaxation of the rings $\rightleftharpoons$ tubulin equilibrium to its proper value in the presence of GTP, and represents the rate-limiting slow dissociation of GDP double rings following the rapid exchange of GTP for bound GDP on tubulin dimers. These data confirm that, in the presence of GTP, the equilibrium between dimers and double rings is less favorable to assembly than in the presence of GDP (Howard & Timasheff, 1986).

Discussion

The ensemble of data presented in this work shows that the actual pathway from disassembling microtubules to double rings at 0 °C is incompatible with either of the theoretical pathways 1 and 2 of Figure 1. Two major findings support this statement. First, the rapid incorporation of a large amount of ^{125}I-labeled tubulin into double rings in the early stages of the disassembly process eliminates the possibility that rings form directly from microtubule protofilaments (pathway 1, Figure 1). While our observations rather favor pathway 2, i.e., disassembly of microtubules into α–β dimers followed by assembly of dimers into rings, the fact that the time course of ^{125}I-tubulin incorporation into rings is complex and at least biphasic, at least in the case of disassembly of MAP-containing microtubules, seems inconsistent with pathway 2 alone and requires the introduction of a third tubulin component into the system, in addition to dimers and double rings. This component would be oligomers of GDP–tubulin in very rapid equilibrium with dimeric tubulin. These oligomers are good candidates for the family of curved linear open assemblies of up to 26 tubulin subunits that are the kinetic intermediates in the isodesmic assembly of tubulin into closed double rings. This interpretation is consistent with the EM observation, in the ring fraction, of short polymers having the same curvature as rings. Comparison of panels A and B of Figure 3 shows that these oligomers, which are labeled within a few seconds, i.e., faster than nucleotide exchange on dimeric tubulin, and practically instantaneously on the time scale of our experiments, represent a more important pool of tubulin in the early stages of the depolymerization process than when the equilibrium between rings and oligomers has been established, which is a characteristic property of a transient species. At the beginning of depolymerization, rings are formed by the association and closure of short oligomers in rapid equilibrium

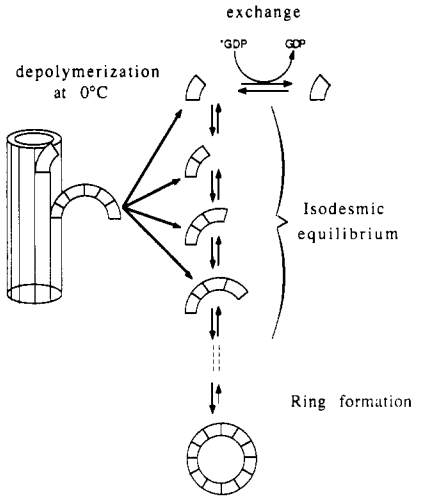

FIGURE 11: Comprehensive kinetic scheme showing the pathway from microtubules to rings at 0 °C: The thick arrows represent rapid reactions, the thin arrows slow reactions. This scheme features the following sequence of events when microtubules are brought to 0 °C: A rapid equilibrium is established between dimers and open oligomer precursors of closed rings. Ring closure is fast, while the opening of the ring is a slow reaction, even slower in the presence of MAPs.

with dimeric tubulin. Had depolymerization given only α–β dimers (pathway 2) that associated rapidly into oligomers, these oligomers would be totally labeled in the early stages. Contrary to this, the oligomer plus ring fraction is only partially labeled initially, and its labeling further increases slowly with time. This indicates that the formed initially short oligomers are, at least in part, direct depolymerization products of microtubules. The isodesmic assembly of tubulin into double rings appears to be very rapid, in all cases more rapid than nucleotide exchange on tubulin. Assuming a plausible range of 1–10 μM^{-1} s^{-1} for the rate constant for the tubulin–tubulin association into oligomers, it becomes clear that the formation of rings from oligomers can be completed within 1 s, while the dissociation of GDP from tubulin has a half-time of 6 s (Engelborghs et al., 1980; Brylawski & Caplow, 1983; Melki et al., 1988). Once the equilibrium between double rings and dimers is established, the rapidly labeled oligomers become a smaller fraction of the population. When the time course of [^{3}H]GDP incorporation into rings was followed, the biphasic incorporation of the label into rings was not observed, since the initial rapid incorporation was probably obscured by the relatively slow exchange of [^{3}H]GDP for unlabeled GDP on dimeric tubulin.

The dissociation of rings into subunits, on the other hand, is a slow reaction which governs the establishment of the equilibrium between dimeric tubulin and double rings. Within the present model, it seems logical to assume that the rate-limiting step in ring dissociation is the opening of the closed structure which leads to the labile open oligomers. The kinetic scheme presented in Figure 11 summarizes these conclusions. This model applies equally to MAP-rich and MAP-free microtubules with the important quantitative difference that ring dissociation is 1 order of magnitude slower when MAPs are bound ($k \sim 10^{-3}$ s^{-1} in the presence of MAPs versus $k \sim 10^{-2}$ s^{-1} in their absence). Ring opening may be kinetically limited, then, by the dissociation of MAPs. A rate constant of the same order of magnitude has been proposed recently by Azhar et al. (1989) for the dissociation of MAPs from microtubules. It is noteworthy that, despite the relatively low temporal resolution of the chromatographic method used here, a semiquantitative estimate of the relative magnitudes of the main kinetic parameters of this system could be obtained.

While this analysis elucidates the mechanism of depolymerization of microtubules at 0 °C, it is quite possible that a different mechanism operates in the isothermal disassembly of microtubules which occurs either upon dilution or in the synchronous oscillations in microtubule assembly observed in buffers in which microtubules are highly dynamic (Carlier et al., 1987b). In particular, the higher lability of lateral interactions relative to longitudinal ones is enhanced at low temperature. Rings, however, still exist at 37 °C, although, in the presence of MAPs, they are less stable at that temperature than at 0 °C (Weisenberg, 1974; Pantaloni et al., 1981). In MAP-free tubulin, their formation is an endothermic reaction (Frigon & Timasheff, 1975), which signifies that the binding of MAPs to tubulin is exothermic. At 37 °C, rings have been shown to dissociate also at a rate (0.02 s^{-1}) 10-fold slower than the exchange of GTP for GDP on α–β-tubulin, this dissociation being the kinetically limiting step in the regeneration of GTP–tubulin from GDP oligomers which determines the period of the oscillations (Melki et al., 1988). In addition, experiments not shown here indicate that at 37 °C as well, the formation of rings from GDP–tubulin is faster than nucleotide exchange on tubulin. All these facts emphasize the agreement between the present data and our model for oscillations (Carlier et al., 1987; Chen & Hill, 1987; Melki et al., 1988), according to which oligomers formed of GDP–tubulin liberated during the rapid depolymerization of microtubules accumulate transiently in antiphase with microtubules, due to their slow dissociation.

The present results have important implications for the structure of tubulin in its various states of assembly and the role of bound nucleotide in microtubule dynamics, as will be developed below. In steady-state microtubules, the bulk of the tubulin exists in the GDP form. This structure is intrinsically unstable (Weisenberg et al., 1976; Carlier & Pantaloni, 1978), and it is maintained by stabilizing caps at the two ends (Carlier & Pantaloni, 1981; Carlier, 1982; Carlier et al., 1984a,b, 1988; Pantaloni & Carlier, 1986). Both the solution equilibrium studies (Howard & Timasheff, 1986) and the structural analysis of species formed in the time course of the cold depolymerization of microtubules have shown that tubulin in the GDP state assembles preferentially in a polar manner into curved protofilaments, with an angle of curvature between consecutive inter α–β-tubulin bonds of ca. 152° (Howard & Timasheff, 1986).[4] In the GTP state, on the other hand, the preferred mode of assembly of α–β-tubulin dimers is close to linear along their longitudinal axis, permitting the formation of two- and three-dimensional structures which are essentially linear along the axes of protofilaments, such as sheets or microtubules. While it is true that steady-state microtubules contain GTP–tubulin only at their ends, these structures are built by the consecutive addition to their ends of α–β-tubulin dimers with the E site occupied by GTP. On hydrolysis of GTP to GDP within the assembled microtubules, the incorporated tubulin α–β dimers remain sterically frozen in what is a metastable state, since GDP–tubulin per se is not capable of such assembly, at least not at any protein concentration accessible in the laboratory. Evidence for the existence of this metastable state is found in the ability of GDP–tubulin in-

[4] This value is deduced from the observation that double rings contain 26 ± 3 α–β-tubulin dimers (Frigon & Timasheff, 1975a). The angle of curvature cited here and in what follows (360°/13 = 27°42′) is taken for purposes of drawing the model, and its absolute value is of no importance to the argument. It contains an uncertainty equal to that in the number of tubulin molecules within the rings. A similar uncertainty must be applied to the interprotofilament curvature within microtubules.

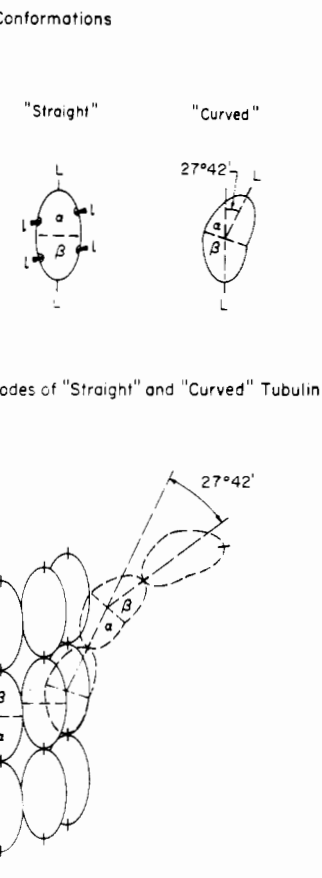

FIGURE 12: Schematic representation of the "straight" and "curved" forms of tubulin. On the drawings, L is a longitudinal bond, and l is a lateral bond within microtubules. Part B shows four protofilaments assembled in microtubular norm. In the assembled structure, the subunits drawn by dashed lines show the effect of the incorporation of tubulin α–β dimers in the curved conformation into a microtubule. As is evident, the first such subunit, when attached to a straight tubulin by one longitudinal bond, is incapable of forming the next such bond within a microtubule.

corporated into microtubules to bind P_i and its structural analogues, the aluminum and beryllium fluoride complexes, while dimeric and oligomeric GDP–tubulins do not bind these analogues (Carlier et al., 1988, 1989). This means that GDP–tubulin exists in different conformations in microtubules, on the one hand, and in α–β dimers or rings, on the other hand. However, our results showing that total dissociation into dimers is not required for the formation of oligomers indicate that the contacts which exist between two tubulins are the same in protofilaments and in rings.

The simplest model which can describe these observations is the existence of tubulin in two structural states which conform to the geometric characteristics of assemblies into microtubules and rings. These are shown schematically in Figure 12A as the "straight" and "curved" conformations. In this model, all the intermolecular contacts and, therefore, all the intrinsic standard free energies of bond formation are kept identical in the various modes of tubulin self-association. As shown in Figure 12A,B, the transition from microtubules to rings would take place simply via a rotation of ca. 28° within tubulin α–β dimers in a direction normal to the axis of the microtubules. Such a motion would coil the protofilaments as shown in Figure 12B. This coiling could be accomplished, for example, by a bending motion at the inter-α–β subunit interface (Figure 12A) which is known to be affected by ligands (Detrich et al., 1982). Engelborghs et al. (1989) have shown that the GDP–GTP exchange is accompanied by a conformational change. The nucleotide bound at the E site acts, then, as an allosteric effector of the observed conformational change which, according to this model, would bend the α–β-tubulin molecule, reorienting inter-tubulin bonds distant from the site of nucleotide binding. In this model, GDP–tubulin exists predominantly in the curved conformation. In the GTP state, tubulin can assemble into either microtubules or double rings, apparently as a result of an equilibrium between two conformations, T ("straight") and T* ("curved") ($K_3 \sim 1$) (Croom et al., 1985; Howard & Timasheff, 1986). Microtubule assembly would occur only from the T, or straight, conformation. This difference in conformation is supported by the observation that, in the isodesmic phase of self-assembly into rings, the equilibrium growth constants are identical for GDP– and GTP–tubulins (Howard & Timasheff, 1986). It is only the final, ring-closing step that is highly favored in GDP–tubulin. This signifies that the assembled chain which exists prior to ring closure is in a geometry (conformation) much more favorable for ring closure in GDP–tubulin than in GTP–tubulin. In GTP–tubulin, the strain imposed in the closure of a 26 α–β-tubulin-containing double ring requires the expenditure of only 0.4 kcal/mol of GTP–tubulin, which is consistent with the measured equilibrium constant for the T $\rightleftharpoons$ T* equilibrium in GTP–tubulin (Howard & Timasheff, 1986). A similar calculation could be made for keeping GDP–tubulin in the microtubule-forming conformation if the microtubule growth equilibrium constant could be measured for GDP–tubulin.

Thermodynamically, the assembly processes of tubulin are based on the ability of tubulin to switch from a "strongly interacting" microtubule-forming state to a "weakly interacting" one (Carlier et al., 1989), with the change localized in the lateral interactions. The thermodynamic linkages between the various reactions are summarized in Figure 13, which incorporates the results both of the present study, of oscillations (Melki et al., 1988), and of ring formation (Howard & Timasheff, 1986). The regulation of these interactions by P_i release, i.e., GTP $\rightleftharpoons$ GDP exchange, could take place either via a conformational change in the site of interaction with a change in the ability to bind to its partner, i.e., a change in the intrinsic standard free energy of bond formation, or by one involving no change in the contact site, but only its displacement in space rendering the contact impossible. The model of Figure 12 conforms to the second alternative. In terms of this model, the strongly interacting state of tubulin would correspond to the straight, microtubule-forming conformation and the weakly interacting state to the curved, ring-forming conformation. Within this model, the intrinsic lability of GDP microtubules is caused not by any change in the intrinsic free energies of intersubunit bond formation, since the chemical nature of the contacts remains unaltered, but by the strain of keeping the polar assembly in linear geometry, when thermodynamically the preferred state is the curved one ($K_1 \ll 1$). The positive free energy increment of this locking by assembly of GDP–tubulin into the "wrong" conformation requires compensation from favorable interactions. These are the locking by neighboring GDP subunits in the two-dimensional lattice within the microtubule core and, at the microtubule ends, maintenance of the polymer by GTP– or GDP–P_i-terminal subunits that interact strongly. Removal of the caps, i.e., elimination of their linked stabilizing free energy, must perforce lead to total depolymerization, since the interaction free energy between GDP–tubulin molecules exposed at microtubule ends would not be sufficient to overcome the strain imposed by the unfavorable geometry. This is exactly what is observed in the catastrophic disassembly char-

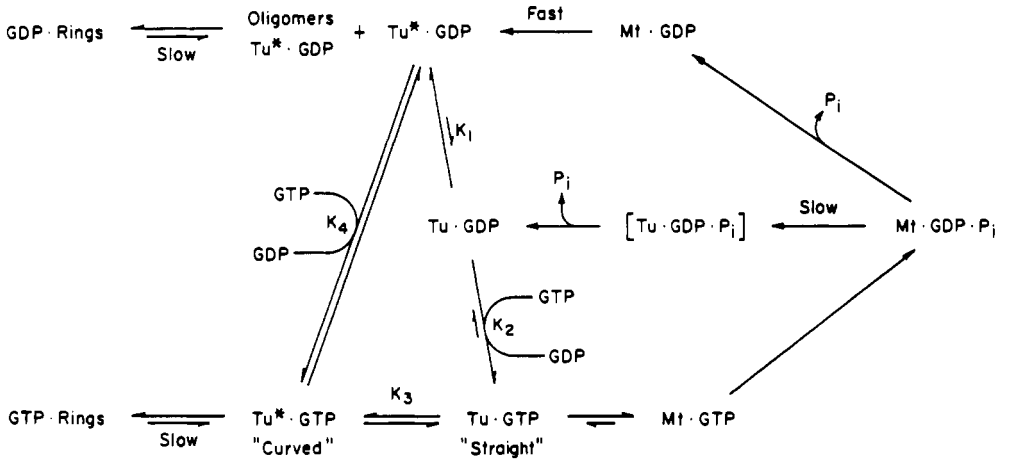

FIGURE 13: Thermodynamic model of the linkages between the polymerizations of tubulin into microtubules and rings and the occupancy of the E site by GTP or GDP.

acteristic of the "dynamic instability" phenomenon (Mitchison & Kirschner, 1984).

The identity of the chemical nature of the binding sites of the curved and straight forms of tubulin is consistent with the known effects of GDP on microtubule formation. (1) The inhibition of microtubule assembly by GDP–tubulin, which blocks elongating sites with an equilibrium constant only 3–5 times lower than for GTP (Zeeberg & Caplow, 1981; Carlier & Pantaloni, 1982; Carlier et al., 1987a), is illustrated in Figure 12B which shows the consequences of introducing a curved tubulin molecule at the growing end of a microtubule. As is seen clearly, this molecule can form only part of the lateral bonds, since the upper, distal end of the dimer can no longer form the proper contacts with its partners in the microtubule. Thermodynamically, this manifests itself as a weakening of the lateral bonds. Propagation of the 27°42′ curvature between consecutive longitudinal bonds and the resulting outward bending of the protofilaments would prohibit the next tubulin molecule to form any lateral bonds within the microtubule, and, thus, growth should stop. (2) The observed low incorporation of GDP–tubulin into microtubules (Hamel et al., 1986; Carlier et al., 1987a) is consistent with the T* $\rightleftharpoons$ T equilibrium being overwhelmingly in favor of the T*, or curved, conformation in GDP–tubulin. This situation is similar to that of the low incorporation of the tubulin–colchicine complex into microtubules (Sternlicht & Ringel, 1979; Saltarelli & Pantaloni, 1983), the binding of which results in a misorientation of the protofilament (Andreu et al., 1983) and weakened lateral bonds (Saltarelli & Pantaloni, 1982) due to the geometric strain. (3) Finally, the inability of GDP–tubulin to nucleate microtubules, while it can participate in elongation under strained conditions, can also find an explanation in the curved mode of protofilament assembly of GDP–tubulin.

A final question to ask is the following: Within this model, what are the interactions which trigger the curved to straight transition? The key interaction must reside in the γ-phosphate of GTP. GTP and GDP both bind to the E site of tubulin with similar affinities ($\Delta G°$ of the binding of GTP is ca. −1 kcal/mol more favorable than that of GDP) (Zeeberg & Caplow, 1979). Recently, Carlier et al. (1988) have shown that the stabilization of microtubules by the caps is dependent on the transient ternary tubulin–GDP–P_i complex which is formed on the postassembly hydrolysis of the E-site GTP. A pertinent observation is the effect of γ-F-GTP on microtubule assembly. This analogue binds to tubulin with an affinity similar to that of GDP (Monasterio & Timasheff, 1987). It mimics GDP in its effects on microtubules in that it acts as a competitive inhibitor of assembly and blocks microtubule growth by binding to the growing end of the structure, and its replacement of GTP induces a conformational change similar to that caused by GDP (Monasterio & Timasheff, 1987; Engelborghs et al., 1989). Yet this analogue contains the same number of phosphates as does GTP. The difference is that γ-F-GTP has only one ionizable hydroxyl on the γ-phosphate. Since GTP, the microtubule-sustaining nonhydrolyzable analogues GMPPCP and GMPPNP, and GDP·P_i, as well as the hydrolyzable analogue GMPCPP,[2] which has been found to induce microtubule assembly (Sandoval & Weber, 1980), all have available two ionizable hydroxyls on the γ-phosphorus, it would appear that the microtubule-forming conformation of tubulin is induced and maintained by the binding of both γ-phosphate hydroxyls in proper position, orientation, and coordination in the E site of tubulin. Violation of this requirement, by removal either of P_i after hydrolysis or of the second ionizable hydroxyl of the γ-phosphate, causes the tubulin structure to collapse into conformations devoid of microtubule-forming ability. Recently, Carlier et al. (1988) have shown that post-GTP hydrolysis replacement of the γ-phosphate of GTP by its analogues BeF_3^- or AlF_4^- (Bigay et al., 1985) results in stable microtubules. This supports further the concept that the switching of the tubulin conformation to one with a strong capacity to form lateral bonds within microtubules is accomplished by the incorporation of a ligand in the proper coordination state into the γ-phosphate binding locus of the E site on tubulin.

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References

Andreu, J. M., Wagenknecht, T., & Timasheff, S. N. (1983) *Biochemistry 22*, 1556–1566.

Azhar, S., Rho, M. B., Wallis, K. T., & Murphy, D. B. (1989) *J. Cell Biol. 107*, 460a.

Bigay, J., Deterre, P., Pfister, C., & Chabre, M. (1985) *FEBS Lett. 191*, 181–185.

Bordas, J., Mandelkow, E. M., & Mandelkow, E. (1983) *J. Mol. Biol. 164*, 89–135.

Brylawski, B. P., & Caplow, M. (1983) *J. Biol. Chem. 258*, 760–763.

Carlier, M.-F. (1982) *Mol. Cell. Biochem. 47*, 97–113.

Carlier, M.-F., & Pantaloni, D. (1978) *Biochemistry 17*, 1908–1915.
Carlier, M.-F., & Pantaloni, D. (1982) *Biochemistry 21*, 1215–1224.
Carlier, M.-F., Simon, C., & Pantaloni, D. (1980) *Biochem. Biophys. Res. Commun. 96*, 1761–1767.
Carlier, M.-F., Hill, T. L., & Chen, Y. (1984a) *Proc. Natl. Acad. Sci. U.S.A. 81*, 771–775.
Carlier, M.-F., Simon, C., & Pantaloni, D. (1984b) *Biochemistry 23*, 1582–1590.
Carlier, M.-F., Didry, D., & Pantaloni, D. (1987a) *Biochemistry 26*, 4428–4437.
Carlier, M.-F., Melki, R., Pantaloni, D., Hill, T. L., & Chen, Y. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5257–5261.
Carlier, M.-F., Didry, D., Melki, R., Chabre, M., & Pantaloni, D. (1988) *Biochemistry 27*, 3555–3559.
Carlier, M.-F., Didry, D., Simon, C., & Pantaloni, D. (1989) *Biochemistry 28*, 1783–1791.
Chen, Y., & Hill, T. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8419–8423.
Detrich, H. W., III, & Williams, R. C., Jr. (1978) *Biochemistry 17*, 3900–3907.
Detrich, H. W., III, Williams, R. C., Jr., & Wilson, L. (1982) *Biochemistry 21*, 2392–2400.
Engelborghs, Y., Robinson, J., & Ide, G. (1980) *Biophys. J. 32*, 440–443.
Engelborghs, Y., Audenaert, R., Heremans, L., & Heremans, K. (1989) *Biochim. Biophys. Acta 996*, 110–115.
Erickson, H. P. (1974) *J. Supramol Struct. 2*, 393–411.
Erickson, H. P., & Pantaloni, D. (1981) *Biophys. J. 34*, 293–309.
Frigon, R. P., & Timasheff, S. N. (1975a) *Biochemistry 14*, 4559–4566.
Frigon, R. P., & Timasheff, S. N. (1975b) *Biochemistry 14*, 4567–4573.
Hamel, E., Batra, J. K., & Lin, C. M. (1986) *Biochemistry 25*, 7054–7062.
Howard, W. D., & Timasheff, S. N. (1986) *Biochemistry 25*, 8292–8300.
Kirschner, M. W. (1978) *Int. Rev. Cytol. 54*, 1–71.
Kirschner, M. W., Williams, R. C., Weingarten, M., & Gerhart, J. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1159–1163.
Mandelkow, E. M., & Mandelkow, E. (1985) *J. Mol. Biol. 181*, 123–135.
Mandelkow, E. M., Harmsen, A., Mandelkow, E., & Bordas, J. (1980) *Nature (London) 287*, 595–599.
Melki, R., Carlier, M.-F., & Pantaloni, D. (1988) *EMBO J. 7*, 2653–2659.
Mitchison, T., & Kirschner, M. (1984) *Nature (London) 312*, 237–242.
Monasterio, O., & Timasheff, S. N. (1987) *Biochemistry 26*, 6091–6099.
Pantaloni, D., & Carlier, M.-F. (1986) *Ann. N.Y. Acad. Sci. 466*, 496–509.
Pantaloni, D., Carlier, M.-F., Simon, C., & Batelier, G. (1981) *Biochemistry 20*, 4709–4716.
Saltarelli, D., & Pantaloni, D. (1982) *Biochemistry 21*, 2996–3006.
Saltarelli, D., & Pantaloni, D. (1983) *Biochemistry 22*, 4607–4614.
Sandoval, I. V., & Weber, K. (1980) *J. Biol. Chem. 255*, 6966–6974.
Scheele, R. B., & Borisy, G. G. (1979) in *Microtubules* (Roberts, K., & Hyams, J. S., Eds.) pp 217–254, Academic Press, New York and London.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.
Spann, U., Renner, W., Mandelkow, E.-M., Bordas, J., & Mandelkow, E. (1987) *Biochemistry 26*, 1123–1132.
Sternlicht, H., & Ringel, I. (1979) *J. Biol. Chem. 254*, 10540–10550.
Vallee, R. B., & Borisy, G. G. (1978) *J. Biol. Chem. 253*, 2834–2845.
Wade, R. H., Pirollet, F., Margolis, R. L., Garel, J. R., & Job, D. (1989) *Biol. Cell 65*, 37–44.
Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.
Weisenberg, R. C. (1974) *J. Supramol. Struct. 2*, 451–465.
Weisenberg, R. C., Deery, W. J., & Dickinson, P. J. (1976) *Biochemistry 15*, 4248–4254.
Zeeberg, B., & Caplow, M. (1979) *Biochemistry 18*, 3880–3886.
Zeeberg, B., & Caplow, M. (1981) *J. Biol. Chem. 256*, 12051–12057.