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Outer Doublet Tubulin Reassembly: Evidence for Opposite End Assembly–Disassembly at Steady State and a Disassembly End Equilibrium†

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ABSTRACT: [^{3}H]GTP exchangeably bound to outer doublet tubulin becomes nonexchangeable with exogenous GTP upon incorporation of the tubulin into microtubules in vitro. We have used this property to study the mechanism of outer doublet tubulin exchange with reassembled microtubules in vitro. At apparent equilibrium, net addition and loss of tubulin occur at opposite ends of the microtubules. The apparent equilibrium is actually a steady-state summation of two different reactions which occur at the opposite microtubule ends, which results in a unidirectional flux of tubulin from microtubule assembly ends to disassembly ends. The similarity of this behavior with that shown previously for bovine brain microtubules in vitro [Margolis, R. L., & Wilson, L. (1978) *Cell 13*, 1] supports our earlier contention that neither the doublet structure nor the stability of outer doublet microtubules in situ is determined solely by the tubulin backbone. Further, the intrinsic assembly–disassembly behavior of tubulins from very diverse sources is strongly conserved. Tubulin loss is readily reversible at the disassembly ends of microtubules polymerized from outer doublet tubulin, indicating that these ends are in equilibrium with tubulin in solution. This does not appear to be the case with bovine brain microtubules that have been assembled in vitro. In addition, the kinetics of podophyllotoxin-induced microtubule depolymerization suggest that addition of podophyllotoxin–tubulin complexes may occur at the microtubule diassembly ends under non-steady state conditions. Thus despite the strong conservation of assembly properties of tubulin from stable sea urchin sperm tail outer doublet microtubules and bovine brain, some differences do exist. These differences may reflect differences in the cellular functions of microtubules.

The in vitro reassembly of tubulin from stable outer doublet microtubules is very similar to that of tubulin from vertebrate brain. In addition, the characteristics of the reassembled microtubules closely resemble those of labile brain microtubules, rather than the stable outer doublet microtubules from which the tubulin was derived (Kuriyama, 1976; Farrell & Wilson, 1978; Binder & Rosenbaum, 1978; Farrell et al., 1979). These results indicate that the polymerization properties of tubulins have been highly conserved and suggest that neither doublet formation nor the stability of microtubules is determined solely by the tubulin subunits (Farrell et al., 1979).

It is possible that other proteins associated with microtubules determine the stability and functions of the microtubules, while chemical heterogeneity among tubulins (Witman et al., 1972; Bibring et al., 1976; Feit et al., 1977; Kobayashi & Mohri, 1977; Marotta et al., 1978; Stephens, 1978) could provide the basis for functional specificity in assembled microtubules.

We have continued our studies on the control of microtubule properties and function by examining the mechanism of tubulin dimer exchange with microtubules reassembled in vitro from outer doublet tubulin. It has been shown previously that tubulin dimer addition and loss occur at opposite ends of microtubules polymerized in vitro from bovine brain tubulin at steady state (Margolis & Wilson, 1978; Wilson & Margolis, 1978). The ability of PLN[1] to poison reassembly of outer doublet tubulin substoichiometrically (Farrell et al., 1979) indicated that a similar assembly–disassembly mechanism existed for microtubules reassembled from outer doublet tubulin.

Determination of the mechanism of tubulin dimer exchange in microtubules reassembled from outer doublet tubulin is of interest for two reasons. First, outer doublet tubulin is derived from microtubules that differ in morphology (Warner, 1972), stability (Behnke & Forer, 1967), and tubulin chemistry (Bibring et al., 1976; Stephens, 1978) from cytoplasmic microtubules such as those derived from vertebrate brain. Although the previous studies have indicated that a number of microtubule properties are not determined solely by the tubulin, a more detailed analysis of the mechanism of tubulin dimer exchange may reveal subtle differences between outer doublet and cytoplasmic tubulins which are functionally important in the assembled microtubules. Secondly, microtubules reassembled in vitro from outer doublet tubulin and

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PLN, podophyllotoxin; CLC, colchicine; MAPs, high molecular weight microtubule associated proteins.

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purified by cycles of assembly–disassembly by using the procedures of Borisy et al. (1974) are greater than 95% tubulin, and appear to be free of associated proteins such as the high molecular weight microtubule associated proteins (MAPs) of vertebrate brain microtubules (Farrell & Wilson, 1978). Microtubules reassembled in vitro from outer doublet tubulin may, therefore, provide a model system, devoid of associated proteins, against which the influence of putative regulatory proteins can be measured.

Materials and Methods

Isolation of Sperm and Preparation of Outer Doublets. Sperm from the sea urchin *Strongylocentrotus purpuratus* was collected either by Pasteur pipet after inducing the animals to spawn with 0.52 M KCl or from whole gonads by the method described by Binder & Rosenbaum (1978). The sperm was stored at −80 °C if not used immediately for preparation of outer doublets.

The procedures for isolating outer doublet microtubules and preparation of assembly-competent tubulin from them have been described in detail elsewhere (Farrell & Wilson, 1978).

Labeling of Microtubules with [^{3}H]GTP. All reassembly experiments employed supernatants from outer doublet microtubules solubilized by sonication in 5 mM Mes, 1 mM EGTA, 1 mM $MgSO_4$, 150 mM KCl, pH 6.7 (reassembly buffer), and centrifuged for 30 min at 200000*g* and 4 °C.

Microtubule formation was initiated by warming the 200000*g* supernatants to 37 °C in the presence of 0.11 mM GTP, 20 mM acetyl phosphate, and 0.05 IUs of acetate kinase (EC 2.7.2.1). The acetate kinase regenerating system maintained the GTP concentration during long-term experiments (MacNeal et al., 1977). Also, it permitted microtubule formation to occur in the low [^{3}H]GTP concentrations used to ensure effective unlabeled GTP chase conditions.

Microtubule labeling was accomplished by the method of Margolis & Wilson (1978). In order to label the microtubules uniformly throughout their lengths, 200000*g* supernatants were added to dried-down [^{3}H]GTP (ICN; 16.7 Ci/mmol; 10–20 μCi/0.2 mL of 200000*g* supernatant) and assembled to steady state at 37 °C. Attainment of steady state was ascertained by light scattering at 350 nm.

In order to label assembling ends, microtubule suspensions at steady state were added to dried-down [^{3}H]GTP (30 μCi/1.0-mL aliquot of microtubules) and incubated at 37 °C for 1 h. Subsequently, a 30-fold excess of unlabeled GTP (3.3 mM) was added to the microtubules to prevent further label incorporation. This concentration of GTP is not inhibitory for microtubules polymerized from outer doublet tubulin (Farrell et al., 1979).

Analysis of Microtubules. The procedure for separating microtubule samples from unincorporated [^{3}H]GTP and analysis of label and protein associated with the microtubule samples was accomplished as described by Margolis & Wilson (1978), except that the 50% and 70% sucrose solutions were made up in 100 mM Mes, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.7 (MEM buffer). Briefly, 300–600-μL aliquots of microtubules, labeled either uniformly or at the assembling ends, were layered onto 4.5-mL 50% sucrose–MEM cushions and centrifuged for 2 h at 40 000 rpm (Beckman, SW50.1 rotor) and 30 °C. The microtubule pellets were depolymerized in 1.0 mL of ice-cold distilled water for 1 h and aliquots removed for counting and protein assay.

Microtubule Length Determination. One-hundred-microliter aliquots of steady-state microtubules were added to 1.0 mL of warm (37 °C) 50% sucrose in MEM. No change in microtubule lengths occurs in this buffer for up to 24 h (Margolis & Wilson, 1978). Samples were immediately prepared for negative stain electron microscopy by the method of Olmsted et al. (1974) and photographed at a magnification of 3300× by using a Philips EM 300 operating at 80 kV. Microtubule lengths were measured directly from photographic prints.

From these measurements, the average length of microtubules in samples was obtained. With these data, the rates of addition or loss of tubulin dimers to microtubules, as the % of microtubule length/h, could be recalculated in terms of μm of microtubule length/h.

Protein Determination. Protein assays were carried out by the method of Lowry et al. (1951) by using bovine albumin as a standard.

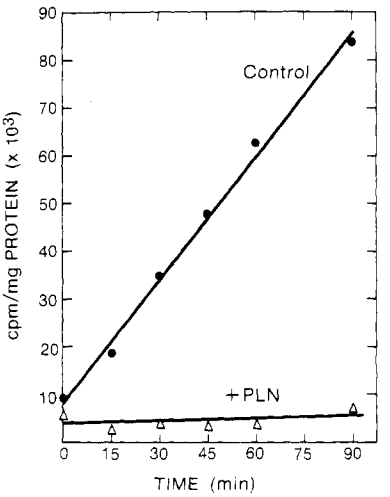

FIGURE 1: Incorporation of [^{3}H]GTP into steady-state microtubules; inhibition by PLN. Outer doublet tubulin was reassembled to steady state in the presence of 0.11 mM unlabeled GTP. At steady state, [^{3}H]GTP (10 μCi/250-μL sample volume; 16.7 Ci/mmol) incorporation in the presence (△) or absence (●) of 5×10^{-5} M PLN was followed for 90 min. Rate of label uptake in the absence of PLN was 7%/h.

Results

Incorporation of [^{3}H]GTP into Microtubules Reassembled to Steady State from Outer Doublet Tubulin. Tubulin isolated from vertebrate brain possesses two binding sites for guanine nucleotides. At one of these sites, the nucleotide is tightly bound (N site), while at the other, the nucleotide rapidly exchanges with guanine nucleotide free in solution (E site) (Jacobs et al., 1974). Although not as extensively studied, a similar situation seems to exist with outer doublet tubulin (Stephens et al., 1967; Stephens, 1969).

Guanine nucleotide bound at the E site of vertebrate brain tubulin becomes nonexchangeable upon dimer incorporation into microtubules (Weisenberg et al., 1976; Margolis & Wilson, 1978). We have tested whether this is also true for outer doublet tubulin. Tubulin in a 200000*g* supernatant was assembled to steady state, as determined by light scattering, and pulsed with [^{3}H]GTP in the presence or absence of 5×10^{-5} PLN. This concentration of PLN totally blocks the reassembly of outer doublet tubulin (Farrell & Wilson, 1978; Farrell et al., 1979).

In the absence of PLN, [^{3}H]GTP was incorporated linearly into steady-state microtubules at a rate of approximately 7%/h. In contrast, label incorporation into PLN-treated microtubules was completely prevented (Figure 1). This demonstrates that exogenous GTP cannot exchange with labeled nucleotide in the microtubules and that GTP uptake into microtubules requires tubulin addition to the microtubules.

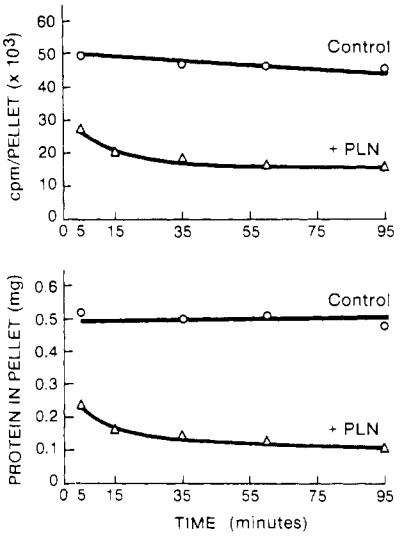

FIGURE 2: Dimer loss from steady-state microtubules in the presence and absence of PLN. Outer doublet tubulin was reassembled to steady state in the presence of 0.11 mM [^{3}H]GTP (25 μCi/4.0 mL sample; 16.7 Ci/mmol). A 30-fold excess of unlabeled GTP in the presence (△) or absence (○) of 5×10^{-5} M PLN was then added and labeled dimer loss followed. The earliest time point which could be measured was at 5 min, the time required to process the samples. Rate of labeled dimer loss from control microtubules was 7%/h. Initial stoichiometry = 0.6 mol of GTP/mol of tubulin dimer in microtubules.

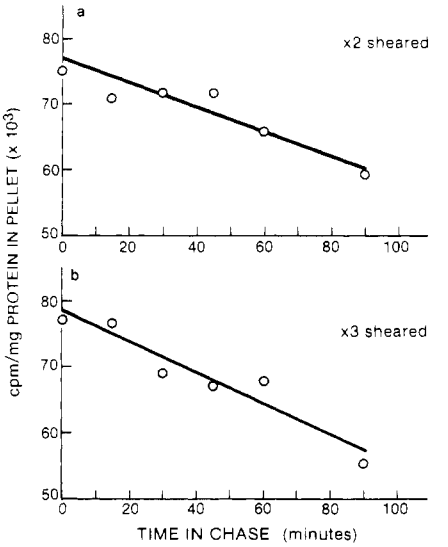

FIGURE 3: Rate of labeled dimer loss from steady-state microtubules as a function of the number of microtubule ends. Outer doublet tubulin was reassembled to steady state in the presence of 0.11 mM [^{3}H]GTP (50 μCi/7.0-mL sample; 16.7 Ci/mmol). At steady state, half the microtubule sample was sheared twice, and the other half three times. Sheared microtubules were obtained by passage of microtubule suspensions through a 6-mL syringe with a 25-gauge needle (×2 sheared = two passages; ×3 sheared = three passages). A 30-fold excess of unlabeled GTP was added, and the rates of labeled dimer loss from the microtubules were followed. Rate of loss from ×2 sheared microtubules = 12.0%/h, corresponding to an average microtubule length of 6.9 μm. Corresponding values for ×3 sheared microtubules = 18%/h and 5.5 μm, respectively. Recalculation of dimer loss rates by length gave a flow rate of 0.87–1.0 μm/h. Initial stoichiometry = 0.58 mol of GTP/mol of tubulin dimer in microtubules.

Loss of Labeled Guanine Nucleotide from Steady-State Microtubules. Outer doublet tubulin in a 200000*g* supernatant was polymerized to steady state in the presence of [^{3}H]GTP to label the microtubules uniformly. At steady state, the labeled microtubules were chased with a 30-fold excess of unlabeled GTP and aliquots removed at time intervals for analysis.

Loss of labeled dimers from the microtubules occurred at approximately the same rate as the rate of dimer incorporation (7%/h, Figure 2). The total mass of assembled polymer, as determined by protein assay, remained constant during the chase period.

Fate of a [^{3}H]GTP Pulse at Steady State. The above data argue that, at steady state, tubulin dimer addition to microtubules occurs at one of the ends of the microtubules, while dimer loss occurs from the opposite ends. If steady state were actually an equilibrium of both microtubule ends with tubulin dimers in solution, neither addition of labeled dimers to, nor loss from, the microtubules should have occurred to any significant extent. We further tested this mechanism as follows. Microtubules at steady state were pulse labeled for 1 h with [^{3}H]GTP, in order to label the assembling A ends, following which a 30-fold excess of unlabeled GTP was added. If net assembly and net disassembly occur at opposite ends of the microtubules, the timing of loss of label will be dependent upon the rate at which the tubulin dimers flux through the microtubules from the pulse-labeled A ends to disassembly (D) ends and upon the average length of the microtubules.

The results showed that no loss of label occurred over 5.5 h subsequent to addition of unlabeled GTP (data not shown). Since the average microtubule length was 8.4 μm, and if opposite end assembly–disassembly occurs, the tubulin dimer flux rate would be 0.87–1.0 μm/h (see below). Therefore, it should take greater than 8 h for the assembly-end pulse to reach the disassembly ends.

Rate of Loss of Labeled Dimers from Steady-State Microtubules as a Function of Microtubule Length. If the separate sites for tubulin dimer addition to, and loss from, microtubules at steady state coincide with the opposite ends of microtubules, the rate of labeled dimer loss, measured as a percentage of total label in the microtubules lost per hour, should be directly proportional to the number of microtubule ends (microtubule number concentration).

To test this, outer doublet tubulin was reassembled to steady state in the presence of [^{3}H]GTP to label the microtubules uniformly. At steady state, the microtubule sample was divided into two aliquots. One aliquot was sheared twice with a syringe, while the other was sheared three times. This resulted in microtubule preparations in which the total mass of assembled polymer was identical, but which differed in microtubule number concentration. Both aliquots were then chased with a 30-fold excess of unlabeled GTP and the rates of loss of labeled dimers from the microtubules followed.

The rate of labeled dimer loss from the microtubules was directly proportional to the microtubule number concentration (assayed by determination of the average microtubule length). Label loss from microtubules sheared twice was 12%/h, corresponding to an average microtubule length of 6.9 μm (Figure 3a). The corresponding values for microtubules sheared three times were 18%/h and 5.5 μm, respectively (Figure 3b). Recalculations of the rates by length gave an intrinsic flow rate for dimers through microtubules of 0.87–1.0 μm/h.

From the above data, we can conclude that, at steady state, net tubulin dimer addition to, and net loss from, microtubules reassembled in vitro from outer doublet tubulin occur at opposite ends of the microtubules.

Influence, on Dimer Loss, of PLN Added to Steady-State Microtubules. PLN poisons the in vitro reassembly of outer doublet tubulin in a substoichiometric manner (Farrell et al., 1979). This requires that PLN binding to microtubule ends prevents significant tubulin dimer addition or loss at the

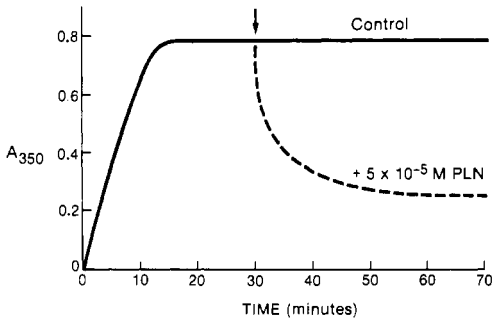

FIGURE 4: Kinetics of PLN-induced depolymerization. Duplicate samples of outer doublet tubulin were reassembled to steady state in the presence of 0.11 mM GTP. The effect of 5×10^{-5} M PLN on steady-state microtubules was monitored relative to an untreated control by light scattering at 350 nm. Arrow indicates time of PLN addition. Protein concentration = 4.5 mg/mL.

poisoned ends (see Wilson & Margolis, 1978, for discussion). We have examined whether PLN poisons both microtubule ends equivalently or whether only the A ends are blocked. If PLN poisons both microtubule ends, dimer loss from these microtubules should not occur. If only the microtubule A ends are poisoned, dimer loss from PLN-treated microtubules should occur initially at approximately 7%/h, the rate of loss from untreated (control) microtubules (Figure 2).

Duplicate samples of outer doublet tubulin were reassembled to steady state, as determined by light scattering at 350 nm, and 5×10^{-5} M PLN was added to one sample, and an equal volume of buffer was added to the second, control sample. The effect of PLN on the assembled microtubules was followed by light scattering (Figure 4). In a second type of experiment, outer doublet tubulin was reassembled to steady state in the presence of [^{3}H]GTP. At steady state, a 30-fold excess of unlabeled GTP was added in the presence or absence of 5×10^{-5} M PLN and the label and protein associated with the microtubules followed with time (Figure 2). The results of both experiments show that dimer loss occurred from PLN-treated microtubules and indicate that the microtubule A ends are blocked by PLN. However, the initial kinetics of dimer loss were more rapid than the 7%/h that would be expected if PLN only blocked assembly at A ends.

By light scattering, dimer loss from PLN-treated microtubules approximated an exponential decay. Complete microtubule depolymerization did not occur, and a new equilibrium appeared to be established (Figure 4). Identical results were obtained when microtubule depolymerization was monitored by labeled dimer loss (Figure 2). Five minutes was required to process the samples; thus, the initial rapid loss of dimers was not measured. By 5 min, a 45% loss of label and of protein from PLN-treated microtubules had occurred.

These data demonstrate that PLN increases the rate of tubulin loss from microtubule D ends. The binding of PLN to free tubulin must be responsible for the effect, suggesting that the microtubule D ends are in equilibrium with tubulin dimers in solution.

Prevention of Labeled Dimer Loss from Microtubule D Ends by Increasing the Free Tubulin Concentration. If tubulin dimers free in solution are in equilibrium with the D ends of steady-state microtubules, dimer loss from microtubule D ends should be prevented by increasing the free tubulin concentration above the apparent K_D for the D ends.

Tubulin was reassembled to steady state in the presence of [^{3}H]GTP, and the microtubules were incubated with 1.6×10^{-6} M CLC for 1 h at 37 °C. We have previously determined that this CLC concentration prevents tubulin addition to the A ends of microtubules by 94%, and, in contrast with PLN,

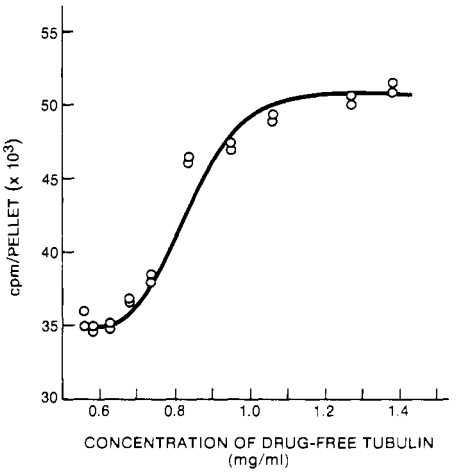

FIGURE 5: Reversal of dimer loss from microtubule D ends. Outer doublet tubulin was reassembled to steady state at 37 °C in the presence of 0.11 mM [^{3}H]GTP (50 μCi/6.0-mL sample; 16.7 Ci/mmol). At steady state, the microtubule A ends were blocked with 1.6×10^{-6} M CLC, and labeled dimer loss from the microtubule D ends was determined after 1 h in the presence of increasing free tubulin concentrations and a 30-fold excess of unlabeled GTP. At this CLC concentration, approximately 24% of the soluble tubulin is complexed with colchicine. This percentage occupancy, while blocking the rate of dimer addition by 94%, does not increase the rate of dimer loss (to be published). We have calculated that the maximum addition of label to the blocked assembly ends in the presence of the unlabeled GTP chase could not exceed 0.002% of the total label in the microtubule.

it does not significantly interfere with tubulin addition to, or loss from, microtubule D ends (to be published). The CLC-blocked microtubules were then incubated with increasing free tubulin concentrations in the presence of unlabeled GTP chases for 2 h, and dimer loss from the microtubules was determined at the end of this period.

Labeled tubulin dimer loss from microtubule D ends was prevented by increasing the free tubulin concentration (Figure 5), demonstrating that the microtubule D ends are in equilibrium with tubulin dimers in solution.

Discussion

Mechanism of Tubulin Dimer Exchange with Steady-State Microtubules. GTP bound to the E site of outer doublet tubulin becomes nonexchangeable upon dimer incorporation into microtubules in vitro (Figure 1). We have used this property to study the mechanism of dimer exchange with steady-state microtubules.

At steady state, the rates of dimer addition to, and loss from, microtubules were approximately equal at 7%/h (Figures 1 and 2). PLN, 5×10^{-5} M, completely prevented dimer addition to microtubules by blocking microtubule ends, but did not stop dimer loss (Figures 1, 2, and 4). Also, loss of labeled dimers did not occur from steady-state microtubules, pulse labeled for 1 h with [^{3}H]GTP, during a 5.5-h chase period (data not shown). Thus, net dimer addition occurs at microtubule ends and net dimer loss occurs at a distant site(s). Since dimer loss from steady-state microtubules was a function of the number of microtubule ends (Figure 3), the site of net dimer loss must be at the opposite end of the microtubule to that for net dimer addition. The rates of outer doublet tubulin dimer addition to, and loss from, steady-state microtubules correspond to tubulin flux rates from microtubule assembly ends to disassembly ends of 0.87–1.0 μm/h.

Reversal of Dimer Loss from Microtubule D Ends: Evidence for a D-End Equilibrium. Labeled dimer loss from steady-state microtubule D ends was prevented by increasing

the free tubulin concentration (Figure 5), demonstrating that both dimer addition and loss can occur at the D ends. It is important to stress that addition of tubulin was carried out along with a large excess of unlabeled GTP, to preclude any possibility of [^{3}H]GTP reutilization in microtubule assembly. In addition, the occupancy of free tubulin dimers by CLC varied between 10 and 24%, which should have completely prevented new microtubule initiation (data not shown).

Prevention of dimer loss was virtually complete at a free tubulin concentration of 1.2 mg/mL (ca. 1.1×10^{-5} M). This concentration must therefore approximate the apparent K_D for dimer addition to microtubule D ends. The steady-state concentration of free tubulin is 0.55–0.70 mg/mL (ca. $0.50–0.64 \times 10^{-5}$ M) at 37 °C (Binder & Rosenbaum, 1978; Farrell et al., 1979). Therefore, at steady state there is net disassembly at the D ends because true equilibrium is never attained.

Depolymerization of Steady-State Microtubules by PLN. The depolymerization kinetics of steady-state microtubules induced by high (5×10^{-5} M) PLN concentration approximated an exponential decay. Complete microtubule depolymerization did not occur and a new equilibrium appeared to be established. It seems correct to refer to the new state as an equilibrium, rather than steady state, since microtubule A ends are completely blocked by this concentration of PLN (Figure 1). Since the PLN concentration was sufficient to complex with all tubulin dimers, both in solution and in microtubules, an equilibrium must exist between microtubule D ends and PLN–tubulin dimer (PD) complexes. If PD complexes could not participate in the D-end equilibrium, complete microtubule depolymerization should have occurred with first-order kinetics. Therefore, at least some addition of PD complexes to microtubule D ends must occur.

The apparent K_D for PD complex addition to D ends must be greater than 1.1×10^{-5} mol/L, the apparent K_D for dimer addition (Figure 5), since PD complex addition to microtubule D ends is thermodynamically less favorable than free dimer addition (Figure 4). Microtubule depolymerization in the presence of high PLN concentrations is therefore initially rapid since the initial concentration of PD complex cannot exceed 0.55×10^{-5} mol/L, the free tubulin concentration at steady state (Farrell et al., 1979), and significant PD complex addition does not occur. The initial rate of dimer loss should, therefore, approximate the intrinsic rate of dimer loss from microtubule D ends. Preliminary data indicate that the half-time for this intrinsic rate of dimer loss is approximately 0.7 min (K. Farrell, K. Sullivan, and L. Wilson, unpublished). Continuing dimer loss increases the PD complex concentration, thereby increasingly favoring PD complex addition to microtubule D ends. This is manifest as a constantly decreasing apparent rate of dimer loss until apparent equilibrium is established.

Possible Relationship between Dimer Exchange Rates and the Composition and Functions of Microtubules. The similar mechanism of dimer exchange with steady-state microtubules assembled from outer doublet and brain tubulins emphasizes our earlier contention that in vitro polymerization properties of tubulins have been highly conserved (Farrell et al., 1979). However, microtubules assembled from these tubulins differ in the rate at which dimers are lost from their D ends in the presence of PLN. In 5×10^{-5} M PLN, microtubules reassembled to steady state from bovine brain tubulin lose dimers from their D ends at the same rate as untreated microtubules (7%/h, Margolis & Wilson, 1978). This suggests that the apparent dimer loss rate from the D ends of these microtubules is relatively independent of the free tubulin concentration. In contrast, microtubules assembled from outer doublet tubulin rapidly lose dimers upon addition of 5×10^{-5} M PLN, which suggests that the dimer loss rate from these microtubules is highly dependent upon the free tubulin concentration. The molecular basis for these differences does not appear to be related to the high molecular weight MAP content of the microtubules. Microtubules reassembled in vitro from outer doublet tubulin do not possess the high molecular weight MAPs of vertebrate brain microtubules (Farrell & Wilson, 1978; Binder & Rosenbaum, 1978). However, we have found that bovine brain MAPs prepared by the method of Sloboda et al. (1976) stimulate the in vitro assembly of outer doublet tubulin and that MAP 2 is preferentially incorporated into the microtubules (Table I). Microtubules reassembled from outer doublet tubulin with bovine brain MAPs have filamentous projections on their outer walls, in contrast to the smooth walls of microtubules polymerized in the absence of brain MAPs (data not shown; Binder & Rosenbaum, 1978). Although bovine brain MAPs physically associate with microtubules polymerized from outer doublet tubulin, we were unable to detect differences in the PLN-induced depolymerization kinetics of these microtubules, compared with microtubules reassembled in the absence of MAPs (data not shown).

We have also found that bovine brain MAPs slow the intrinsic flux rate of dimers in microtubules polymerized from outer doublet tubulin threefold, compared with control microtubules not containing MAPs (Table I). This raises the question of whether this decrease in the tubulin flux rate is significant for the regulation of microtubule properties. In the presence of PLN, microtubules reassembled from outer doublet tubulin lose dimers from their D ends at the same rate, irrespective of the presence or absence of brain MAPs, and this fact suggests that this decrease in the flux rate is not significant, at least for microtubule stability to PLN. Further clarification of the possible roles of MAPs on microtubule functions might be obtained by examining the tubulin flux

Table I: Effect of Bovine Brain MAPs on Steady State Flux Rates of Microtubules Reassembled from Outer Doublet Tubulin[a]

assembly conditions	microtubule composition (%) MAP 1	MAP 2	tubulin	intrinsic tubulin flux rate at steady state (μm/h)
outer doublet tubulin	0	0	100	1.49[c]
outer doublet tubulin + bovine brain MAPs[b]	0.5	8.4	91.1	0.50[c]
bovin brain tubulin	3.6	18.9	77.5	0.6–0.7[d]

[a] Outer doublet tubulin was purified by 1 cycle of assembly–disassembly (Farrell & Wilson, 1978) and contained no associated proteins. Bovine brain tubulin was purified by 3 cycles of assembly–disassembly (Borisy et al., 1974). MAPs were prepared using phosphocellulose chromatography (Sloboda et al., 1976) from bovine brain microtubules purified by 3 cycles of assembly–disassembly. The high molecular weight MAPs 1 and 2 formed 95% of the MAP fractions and tubulin was not present. Microtubules were reassembled to steady state at 37 °C by using the acetate kinase regenerating system of MacNeal et al. (1977) and separated from unpolymerized proteins by centrifugation through a 50% sucrose cushion (150000g, 2 h, 30 °C). Microtubule pellets were analyzed by planimetry of 6% NaDodSO$_4$–polyacrylamide gels (Weber & Osborn, 1969) and areas under peaks expressed as a % of total scan area. Tubulin flux rates of microtubules reassembled in the presence and absence of brain MAPs were determined as described under Materials and Methods. [b] MAPs were added to a final MAPs/tubulin weight ratio of 0.3. This ratio was determined to yield maximal stimulation of initial rates and final extents of assembly by light scattering at 350 nm. [c] Mean of two experiments. [d] Tubulin flux data from Margolis & Wilson (1978).

rates and drug stabilities of vertebrate brain microtubules reassembled in the presence and absence of the MAPs.

It has been suggested that the intrinsic tubulin flux rates of microtubules are an important factor in determining microtubule stability to antimitotic drugs in vitro (Wilson & Margolis, 1978). The fact that microtubules reassembled from bovine brain tubulin and from outer doublet tubulin with brain MAPs have similar flux rates but different stabilities to PLN suggests that the magnitude of the intrinsic rate constant for tubulin dimer loss at the microtubule D ends may also be an important consideration.

In conclusion, although the polymerization properties of bovine brain and outer doublet tubulins have been conserved, differences in the behavior of microtubules assembled from these tubulins are apparent. Since these tubulins were derived from microtubules with diverse properties and unique associated proteins, elucidating the molecular basis for the differences between the reconstituted microtubules may be important for understanding the physiological control of microtubule behavior.

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