Concerning the Chemical Nature of Tubulin Subunits That Cap and Stabilize Microtubules†

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ABSTRACT: There is no definitive evidence on the nature of the cap at microtubule ends that is responsible for dynamic instability behavior. It was, therefore, of interest that steady-state microtubules assembled in 20 mM P_i buffer and pulsed for 15−60 min with [γ-^{32}P]GTP contained approximately 26 [^{32}P]P_i/microtubule [Panda et al. (2002) *Biochemistry 41*, 1609−1617]. It was concluded that microtubules are capped with a tubulin−GDP−P_i subunit at the end of each its 13 protofilaments and that this is responsible for stabilizing microtubules in the growth phase. Also, because microtubules with [^{32}P]P_i were isolated despite the presence of 20 mM P_i, it was concluded that P_i in terminal tubulin−GDP−P_i subunits does not exchange with solvent. These observations are inconsistent with our finding that tubulin−GDP−P_i subunits do not stabilize microtubules and with evidence that the nucleotide, and presumably also P_i, in subunits at microtubule ends exchanges with solvent. We have resolved this discrepancy by finding that during the pulse period the added [^{32}P]GTP was almost quantitatively hydrolyzed. The so-formed [^{32}P]P_i labeled the 20 mM P_i buffer, and this exchanged into tubulin−GDP subunits in the core of the microtubule. Evidence for this was our finding of virtually identical [^{32}P]P_i in microtubules pulsed with [^{32}P]GTP with a specific activity that varied 11-fold by using either 100 or 1100 μM GTP in the reaction. Label uptake was insensitive to the [^{32}P]GTP specific activity because in both cases hydrolysis generated 20 mM [^{32}P]P_i with a virtually identical specific activity. Also, approximately 0.4 mol of [^{32}P]P_i /tubulin dimer was found in microtubules when steady-state microtubules in 20 mM P_i were pulsed with a trace amount of [^{32}P]P_i. This stoichiometry is consistent with a 25 mM K_d previously reported for P_i binding to tubulin−GDP subunits in microtubules. It is concluded that, under the conditions used for the [^{32}P]GTP pulse labeling, ^{32}P was incorporated into the entire microtubule from [^{32}P]P_i released into the solution, rather than into a tubulin−GDP−P_i cap, from [^{32}P]GTP. Thus, there is no evidence that tubulin−GDP−P_i subunits accumulate in and stabilize microtubule ends.

It is not known how the structure differs in tubulin subunits at the ends of shrinking and growing microtubules undergoing dynamic instability behavior (*1*). Despite a lack of evidence, several textbooks (*2*−*4*) describe growing microtubules as containing a large core of unstable tubulin−GDP subunits, capped with stable tubulin−GTP[1] subunits at both ends. Evidence for an inhomogeneity between the end and the body of the microtubule was the observation of a several second lag for attaining a maximum disassembly rate, after the temperature of steady-state microtubules was rapidly decreased from 36 to 34 °C (*5*). Identifying the inhomogeneity in the structure of microtubules remains a major question for understanding microtubule dynamics.

The assumption that growing microtubules can be made stable by a tubulin−GTP cap is consistent with the lower rate of dissociation of tubulin−GTP subunits, compared to tubulin−GDP subunits. The former rate, determined from the *Y*-axis extrapolation of the rate of microtubule growth as a function of the tubulin−GTP concentration, is only 23−44 s^{-1} (*6*) or 0.37−1.1 s^{-1} (*1*). In comparison, the rate of dissociation of tubulin−GDP subunits is 733−915 s^{-1} (*6*) or 212−340 s^{-1} (*1*) or 880−1210 s^{-1} (*7*).

Additional evidence for stabilization with GTP is that the rate of dissociation of subunits containing the GTP analogue GMPCPP is only 0.1 s^{-1} (*8*) or 0.118 s^{-1} (plus end) and 0.065 s^{-1} (minus end) (*9*). Furthermore, because stabilization of microtubules by tubulin−GMPCPP subunits is proportional to the 13−14th power of the mole fraction of subunits containing this GTP analogue (*9*), it appears that a monolayer GMPCPP cap is necessary and sufficient to stabilize microtubules. Quantitative analysis of the fluorescence of microtubules pulsed with rhodamine-labeled tubulin−GMPCPP subunits indicated that a microtubule end can be stabilized by as few as 14 tubulin−GMPCPP dimers/microtubule (*10*).

Evidence for the structural basis for the effect of GTP was a change in the moire pattern when GDP in microtubules

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[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GTP, guanosine 5′-triphosphate; MAPs, microtubule-associated proteins; LGPEN buffer, 100 mM sodium L-glutamate, 20 mM sodium phosphate, 1 mM EGTA, and 0.02% sodium azide, pH 6.75; Tu−GTP, tubulin with GTP at the exchangeable (E) site; Tu−GDP, tubulin with GDP at the exchangeable (E) site; Tu−GDP−P_i, tubulin with GDP and orthophosphate bound at the exchangeable (E) site; GAP, GTPase activating protein; GMPCPP, guanylyl α,β-methylenediphosphonate.

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was replaced with GMPCPP; this was consistent with a 1.5 Å increase in the subunit size along the axis of the microtubule (*11*). The different structure of microtubule ends with GTP and with GDP can be detected by kinetochores in vitro, which bound preferentially to GTP microtubules compared to GDP microtubules (*12*).

There is substantial evidence that the GTP cap cannot be large. The virtual identity of the pre-steady-state rates of microtubule assembly and of GTP hydrolysis provides strong evidence against the accumulation of significant GTP at the ends of growing microtubules (*13*−*15*). Even at very high subunit addition rates, the rate of hydrolysis is equal to that for subunit addition. Further evidence against a large GTP cap is a <1 s lag for start of disassembly following dilution of microtubules into tubulin-free buffer (*16*). On the basis of a rate of tubulin−GTP subunit dissociation equal to 23−44 s^{-1} (*6*) or 0.37−1.1 s^{-1} (*1*), it was concluded the GTP cap contains no more than 23−44 subunits, or 0.37−1.1 tubulin−GTP subunits.

Failure to obtain evidence for a significant GTP cap led to the suggestion that growing microtubules are stabilized by a monolayer of tubulin−GTP subunits that escapes detection by chemical or kinetic methods. One possible mechanism for generating a monolayer cap would be to have GTP hydrolysis induced in terminal tubulin−GTP subunits when these contact a tubulin subunit that adds to the end (*17*−*19*). According to this model an adding tubulin−GTP subunit serves as a GAP for hydrolysis of nucleotide in the subunit to which it adds. Structural studies (*20*−*22*) suggest GAP activity is in the α-subunit, with the active site provided by a loop between helices 7 and 8, with major contribution by conserved residues D251 and E254. Addition to the microtubule end brings the loop into contact with GTP in the *β*-subunit at the terminus of the plus end of microtubules. In support of the GAP model, mutations of residues 251 and 254 were dominant lethal in yeast (*22*), with a phenotype that included abnormally stable microtubules that presumably contained GTP. It was, however, not possible to prove the proposed model because the dominant lethal phenotype conferred by the mutation precluded isolating tubulin for measuring GTPase activity. Note that although the proposed mechanism can maintain a monolayer GTP cap at the plus end, the minus end is not expected to have a GTP cap (*20*). Rather, hydrolysis is predicted in the adding subunit because its GTP makes contact with the putative GAP loop in the α-subunit at the microtubule end. Nevertheless, because the minus end has dynamic instability behavior (*6*, *23*), it can be concluded this end, too, is capped while in the growth phase.

In a recent model to account for dynamic instability growing microtubules are stabilized by tubulin−GDP−P_i at ends, and disassembly occurs when these are replaced by tubulin−GDP subunits. Evidence that tubulin−GDP−P_i is stabilizing was that BeF_3, which binds to microtubules competitively with P_i, decreased the dimer dissociation rate (*25*). Although it was assumed that tubulin−GDP−BeF_3 and tubulin−GDP−P_i subunits behave similarly, with G-proteins the properties of the GDP−BeF_3 complex resemble those of the G-protein bound to GTP (*26*−*28*). Thus, stabilization by tubulin−GDP−BeF_3 cannot be taken as evidence for stabilization by tubulin−GDP−P_i. Evidence that tubulin−GDP−P_i subunits do not stabilize microtubules is the lack of stabilization of microtubules by 167 mM, 50 mM (*7*, *29*), or 80 mM P_i (*30*). Also, 50 mM P_i added to microtubules stabilized by 100 *μ*M BeF_3 reversed the stabilization by displacing BeF_3 (*29*). Finally, proof that significant amounts of P_i do not accumulate in growing microtubules came from comparing the rates of assembly and of P_i release from growing microtubules. Direct observation of the time course of nucleated assembly indicated that the P_i release was closely coupled to microtubule elongation, even during the initial stages of assembly when uncoupling of subunit addition and GTP hydrolysis would be most likely to occur (*14*).

Results from recent studies (*31*) were taken as evidence for a unique population of P_i-binding tubulin subunits at microtubules end that would go undetected in the above-mentioned fluorometric assay for P_i release. Terminal subunits were found to bind P_i more tightly than subunits within the microtubule (*31*), as evidenced by finding 13 tubulin−GDP-[^{32}P]P_i subunits/microtubule end, in steady-state microtubules that were pulsed for 15−60 min with [*γ*-^{32}P]GTP. Surprisingly, unlike internal subunits, which exchange with P_i in solution (*25*), microtubules with tubulin−GDP−[^{32}P]P_i were isolated from buffer containing 20 mM P_i (*31*). The nonexchangeabilty of P_i in terminal subunits was also surprising because we found in an earlier study that subunits at microtubule ends undergo nucleotide dissociation to form apotubulin subunits, as well as nucleotide exchange (*32*). Consistent with this, fluorescent beads linked to GTP can bind to tubulin subunits at the plus end of MTs (*33*), indicating that the E-site is open for exchange. Because of these discrepancies we have further analyzed uptake of [^{32}P]P_i from a [*γ*-^{32}P]GTP pulse. We conclude that this was derived from uptake of approximately 0.5 mol of [^{32}P]P_i/tubulin dimer along the length of the microtubule, rather than from [^{32}P]GTP into 13 subunits at microtubule ends.

MATERIALS AND METHODS

Three-cycle beef brain microtubule protein was prepared as described elsewhere (*31*) or was obtained as a generous gift from Dr. Leslie Wilson. Gel analysis showed the protein was approximately 70% tubulin, with numerous high molecular weight MAPs. Defrosted protein was diluted into LGNPEM and incubated on ice for 15 min before being centrifuged at 38000*g* for 15 min at 4 °C to remove protein aggregates. *Escherichia coli* acetate kinase was from Sigma (A2384), and at a concentration 10-fold higher than used in the pulse reactions with 100 *μ*M [*γ*-^{32}P]GTP (see below), this cleaved only 1% of [*γ*-^{32}P]GTP in 30 min.

Steady-state microtubules were pulsed with [*γ*-^{32}P]GTP, as described previously (*31*). Pulse reactions were also done with [^{32}P]P_i (Amersham), which was filtered immediately before use to remove a microparticulate impurity (*34*) that pelleted though the sucrose cushion used to isolate microtubules. In the pulse studies ∼3.5 mg of microtubule protein was assembled to steady state at 30 °C in a 700 *μ*L reaction mixture containing LGNPEM buffer, along with 10 mM acetyl phosphate and 0.1 IU/mL acetate kinase and either 100 or 1100 *μ*M GTP; GTP was 100 *μ*M in earlier studies (*31*). After 20 min, microtubules were sheared by six passes through a 22-guage needle and incubated for an additional 20 min to reestablish a steady state. A 75 *μ*L aliquot of each

of the two reaction mixtures was removed immediately after shearing, to be used to measure the GTPase rate. When the microtubules had reestablished a steady state, [γ-^{32}P]GTP was added, 0.33 μCi for the GTPase reaction and 6.5 μCi for microtubules that were to be isolated. After 38 min microtubules were isolated by centrifuging 350 μL of the reaction mixture through a 4.3 mL cushion of LGNPEM buffer with 50% sucrose. Centrifuging was for 2 h at 44000 rpm in a SW65 rotor at 30 °C. In control reactions with [^{32}P]-P_i, but without microtubules or tubulin, only 0.0033% of the label was pelleted. Thus, there is little contamination of the pellet with the radioactive supernatant. Microtubules in the pellet (approximately 1.2 mg) were disassembled in 200 μL of PGNPEP buffer for 20 min at 0 °C, and the amount of ^{32}P was determined in an aliquot of the reaction both before and after precipitating the protein with 0.7 M $HClO_4$. Acid-soluble radioactivity corresponds to [^{32}P]P_i (*31*), whereas total radioactivity is from [^{32}P]P_i and from ^{32}P covalently bound to MAPs. The distribution of radioactivity was also determined using a Phosphorimager to detect the label in a 7.5% cross-linked acrylamide−SDS electrophoretic gel. Electrophoresis of a 30 μL aliquot of the pelleted microtubules was interrupted when the dye front had migrated about 60% of the length of the gel, so that [^{32}P]P_i was not lost. The gel was wrapped in a single layer of plastic wrap before being placed on a Phosphorimager plate. The identity of the ^{32}P in microtubules that had been pulsed with [^{32}P]P_i was determined by thin-layer chromatography on PEI−cellulose, with development with 1 M LiCl and 1 M formic acid (*31*).

The rate of GTP hydrolysis was determined by quenching a 7 μL aliquot of the reaction in 500 μL of a cold solution containing 1 M hydrochloric acid, 4% perchloric acid, 180 μM P_i, and 10 mM ammonium molybdate. [^{32}P]P_i was extracted into 1 mL of organic reagent (cyclohexane, isobutyl alcohol, and acetone acid−molybdate mixture at a ratio of 50:50:10:1). After phase separation was complete the radioactivity in the organic phase was measured in a Beckman scintillation counter.

RESULTS AND DISCUSSION

There were two elements in the earlier study of [^{32}P]P_i incorporation into steady-state microtubules that suggested that label uptake may be derived from [^{32}P]P_i rather than [^{32}P]GTP, as had been suggested (*31*). First, label uptake was found with tubulin containing MAPs with GTPase activity that apparently resulted from the sequential action of protein kinase(s) and protein phosphatase(s). Evidence for high activity in this kinase/phosphatase cycle was that 12−62% of the label in isolated microtubules was covalently bound to high MW MAPs after steady-state microtubules were pulsed with 100 μM [γ-^{32}P]GTP for 15 min (*31*). This uptake of label occurred despite the fact that the microtubules had been at steady state for 40 min in the presence of nonradioactive GTP, as well as the fact that the tubulin and MAPs had been exposed to GTP for several hours during purification of the protein. The important point is that the [γ-^{32}P]GTP consumed by the kinase/phosphatase cycle would be replaced by nonradioactive GTP, by action of the acetate kinase/acetyl phosphate regenerating system, so the specific activity of the [γ-^{32}P]GTP would fall. At the same time, [^{32}P]-P_i formed from [γ-^{32}P]GTP would progressively increase the specific activity of the 20 mM P_i.

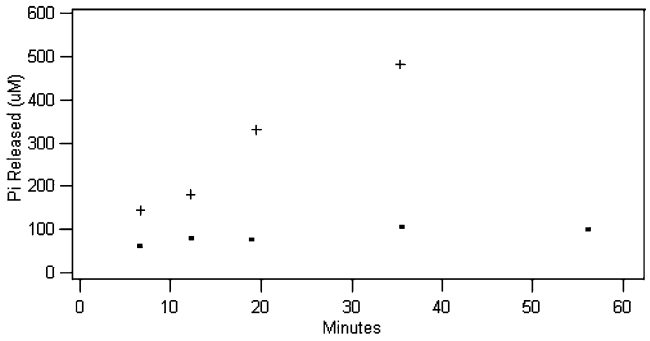

FIGURE 1: Hydrolysis of GTP by steady-state microtubules during a pulse with [γ-^{32}P]GTP. The [γ-^{32}P]GTP concentration was 100 μM (+) or 1100 μM (■); protein was provided by Panda et al. (*31*).

The other reason for suspecting that label uptake may be derived from [^{32}P]P_i rather than [^{32}P]GTP was a coincidence between the ratio of the P_i/GTP concentrations and the ratio of nonterminal tubulin subunits/terminal subunits for the microtubules used in the experiment (*31*). The former ratio was 20 mM/100 μM = 200, which was almost identical to the ratio of terminal and nonterminal subunits in the 3 μm mean-length microtubules in the reaction (i.e., 5000/26). The coincidence of the two ratios means that approximately the same number of counts will be found in microtubules if 26 terminal subunits are labeled from [^{32}P]GTP or if 5000 tubulin−GDP subunits are labeled from 20 mM [^{32}P]P_i.

We determined the source of label uptake into steady-state microtubules by measuring the GTPase rate with an assay for [^{32}P]P_i. Release of [^{32}P]P_i from [^{32}P]GTP decreases the specific activity of the [^{32}P]GTP and increases that in the 20 mM pool of [^{32}P]P_i. GTP hydrolysis was previously analyzed with a malachite assay that measures nonradioactive P_i (*31*); therefore, changes in the specific activity could not be detected. Also, in this colorimetric assay the zero-time reading is expected to be extraordinarily high from the 20 mM P_i in the buffer. Since the GTP concentration was only 100 μM, quantitative hydrolysis of the GTP will yield a 5% increase in the colorimetric signal. The reported rate equal to <2%/h (*31*) corresponded to a <0.01% change in the P_i concentration.

We found that the initial steady-state GTPase rate with 100 μM [γ-^{32}P]GTP was 4 μM/min (27% hydrolysis in 6.5 min) with protein prepared in our laboratory and ≥10 μM/min (Figure 1, 62% hydrolysis at 6.5 min) with protein prepared by Panda et al. (*31*). The rate measured here is in reasonable agreement with that found previously (*35*) where the steady-state GTPase rate with microtubules formed with 40 μM tubulin was about 3 μM/min. This rate was not appreciably different with and without 3.4 M glycerol, so that dynamic instability, which is muted in the presence of MAPs and glutamate (*31*), apparently does not make a significant contribution to the GTP hydrolysis rate.

The protein(s) catalyzing GTP hydrolysis was (were) not saturated with GTP at 100 μM, and the initial rates with 1100 μM [γ-^{32}P]GTP increased to 9 μM/min with protein prepared in this laboratory and 22 μM/min with protein provided by Panda et al. With protein prepared by Panda et al. (*31*) 100 μM [γ-^{32}P]GTP was about 75% hydrolyzed in 15 min and 100% hydrolyzed in 60 min, so that virtually all of the radioactivity was in [^{32}P]P_i at both 15 and 60 min.

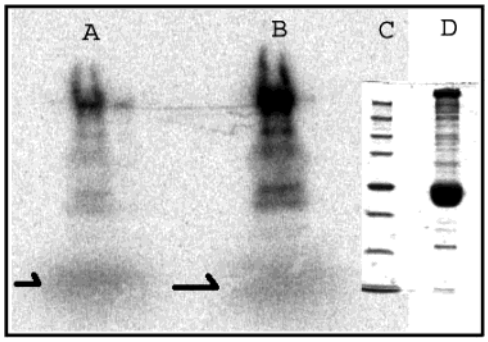

FIGURE 2: Distribution of ^{32}P in microtubules after a 40 min pulse with 100 μM (B) or 1100 μM (A) [γ-^{32}P]GTP. The total label in microtubules was about 1.6-fold greater with 100 μM nucleotide; however, the amount of label in [^{32}P]P_i (arrows) was about equal (15% total with 100 μM and 28% total with 1100 μM [γ-^{32}P]GTP). Lanes C and D are Coomassie-stained gels from the microtubule pellet and molecular mass standards [250, 150, 100, 75, 50 (dark), 37, 25, 15, and 10 kDa].

The earlier observation of identical uptake of [^{32}P]P_i after a 15 and a 60 min pulse (*31*) is consistent with [^{32}P]P_i providing the source of the label.

Additional evidence that [^{32}P]P_i uptake into steady state comes from [^{32}P]P_i, rather than from [γ-^{32}P]GTP, was obtained by varying the specific activity of the [γ-^{32}P]GTP. Reducing this 11-fold by increasing the GTP to 1100 μM gave microtubules with about 37% less radioactivity; however, the amount of [^{32}P]P_i was similar (Figure 2). Note that when microtubules were isolated at 40 min in the reaction with 1100 μM [^{32}P]GTP, only about half of the ^{32}P was in the pool of [^{32}P]P_i (Figure 1), whereas with 100 μM [^{32}P]-GTP all of the ^{32}P was in the 20 mM P_i pool. It is suggested that experimental error obscured the effect of the 50% lower [^{32}P]P_i specific activity with 1100 μM GTP. Also, although microtubule isolation was started at 40 min, it may take some time before microtubules are separated from the pool of radioactive substrates. The greater total radioactivity found in microtubules pulsed with 100 μM [γ-^{32}P]GTP came from label uptake into MAPs (Figure 2) at the higher [^{32}P]P_i specific activity.

Virtually identical results were obtained from measurements of the fraction of the total ^{32}P bound to the microtubule that corresponded to [^{32}P]P_i when this was determined from measurement of the perchloric acid-soluble radioactivity and from the [^{32}P]P_i band after gel electrophoresis (Figure 2). In pulse reactions initiated with 100 μM [γ-^{32}P]GTP the average stoichiometry for label uptake into microtubules was 0.55 mol of [^{32}P]P_i /tubulin dimer (Table 1). This was calculated on the assumption that label was derived from 20 mM [^{32}P]-P_i containing all of the radioactivity that was initially present in added [γ-^{32}P]GTP. The stoichiometry when the pulse was with 1100 μM [γ-^{32}P]GTP is less certain, since not all of the label was released by hydrolysis. However, the stoichiometry calculated (Table 1), assuming that 50% of the [γ-^{32}P]-GTP had been hydrolyzed when microtubules were isolated (see Figure 1), was in reasonable agreement with that measured at the lower equal [γ-^{32}P]GTP concentration. On the basis of a K_d equal to 25 mM for P_i binding to microtubules (*24*), the stoichiometry is expected to equal 0.44 for binding 20 mM P_i to microtubules.

Evidence that label uptake was from [^{32}P]P_i in reactions with [γ-^{32}P]GTP was also obtained using [^{32}P]P_i instead of [γ-^{32}P]GTP in the pulse. With tubulin prepared in this laboratory as well as with protein provided by Panda et al. (*31*), about 0.4 mol of P_i was found in pelleted microtubules (Table 1). The radiolabel was shown to be [^{32}P]P_i by thin-layer chromatography; SDS gel analysis of the pellet showed that there was no label incorporated into proteins. It was previously shown that radiolabel is in [^{32}P]P_i in microtubules pulsed with [γ-^{32}P]GTP (*31*). It is noted that our finding that [^{32}P]P_i that has entered microtubules by an exchange reaction is not displaced by the 20 mM P_i in the 50% sucrose cushion suggests the protein conformation in the region of the E-site is altered by sucrose. In support of this, we had found that a high concentration of glycerol alters the E-site of tubulin in microtubules so that it readily hydrolyzes the γ-phosphate in bound GMCPP (*9*).

Table 1: Tubulin and [^{32}P]P_i in Microtubules after Pulsing with [γ-^{32}P]GTP and with [^{32}P]P_i

expt	^{32}P source	nmol of [^{32}P]P_i/nmol of tubulin (stoichiometry)[a] with 100 μM [γ-^{32}P]GTP	nmol of [^{32}P]P_i/nmol of tubulin (stoichiometry)[a] with 1100 μM [γ-^{32}P]GTP	nmol of [^{32}P]P_i/nmol of tubulin (stoichiometry)
1	[γ-^{32}P]GTP	4.19/10.00 (0.42)	5.50/8.85 (0.62)	
2	[γ-^{32}P]GTP	3.44/5.12 (0.67)	4.57/7.07 (0.65)	
3[b]	[γ-^{32}P]GTP	5.11/8.27 (0.62)	2.50/8.27 (0.30)	
4	20 mM [^{32}P]P_i			9.91/15.27 (0.65)
5	20 mM [^{32}P]P_i			6.74/14.36 (0.47)
6	20 mM [^{32}P]P_i			7.15/15.80 (0.45)
7[b]	20 mM [^{32}P]P_i			2.63/8.40 (0.31)
8[b]	20 mM [^{32}P]P_i			1.95/8.61 (0.23)
9[b]	20 mM [^{32}P]P_i			2.41/9.36 (0.26)

[a] The specific activity of the [^{32}P]P_i was calculated on the assumption that, in reactions with high specific activity [γ-^{32}P]GTP (100 μM nucleotide), all of the initial radioactivity in GTP was incorporated into 20 mM P_i and that, with low specific activity [γ-^{32}P]GTP (1100 μM nucleotide), half of the radioactivity was in 20 mM P_i. The lower [^{32}P]P_i specific activity in the latter reactions resulted because only about 50% of the nucleotide was cleaved when the microtubules were isolated (Figure 1). [b] Experiments with protein were provided by Herb Miller (*31*).

Drug and Analogue Effects on Label Uptake. It was reported (*31*) that colchicine and vinblastine reduced the uptake of [^{32}P]P_i from 25.5 to 10.5−12.8/microtubule when steady-state microtubules were pulsed with [γ-^{32}P]GTP. This was suggested to result from a reduced rate of tubulin subunit addition to microtubule ends so that only about 12 subunits were added during the 20 min pulse with [^{32}P]GTP (*31*). Alternatively, the drugs induced a protein conformation change that altered the stoichiometry for P_i binding to the cap. In light of our evidence that label uptake results from [^{32}P]P_i binding to microtubule subunits at the site normally occupied by the γ-phosphate moiety of GTP, we suggest that the reduction with drugs resulted because these decreased the rate of [^{32}P]GTP hydrolysis from subunit addition to microtubule ends and/or by the kinase/phosphatase reactions. Reduced hydrolysis would provide less [^{32}P]P_i to label the P_i pool for uptake into microtubules. Alternatively, incorporation of tubulin−colchicine subunits into microtubules (*36*) may have increased the K_d for P_i binding. The effect of vinblastine may have resulted from its forming nonmicrotubule polymers (*37*) with an increased K_d for P_i.

It was also found that uptake of [^{32}P]P_i into steady-state microtubules was increased about 1.6-fold by 5 mM NaF and 70 μM BeF_3 or AlF_3 (*31*). This increase was not accounted for. For the mechanism proposed here, it is not

expected that 70 μM BeF_3 will displace significant [^{32}P]P_i from microtubules since its apparent K_d is equal to 700 μM (*28*). We are not able to account for the increased binding of [^{32}P]P_i seen with NaF, but with BeF_3 and with AlF_3 there may be a cooperative component to the binding of P_i, so binding of BeF_3 enhances binding of P_i to neighboring subunits. This would be similar to the enhanced binding of oxygen to hemoglobin in the presence of a low concentration of CO (*38*).

Nature of the Microtubule Stabilizing Cap in Other Systems. Although our results with porcine and bovine brain tubulin here and earlier (*9*, *28*) rule out a stabilizing tubulin−GDP−P_i cap, this or some other mechanism for stabilizing growing microtubules may hold for tubulin from other species. In microtubule assembly with chick brain tubulin the hydrolysis of GTP significantly lags microtubule assembly, so that significant tubulin−GTP accumulates in microtubules (*39*). Thus, a GTP cap mechanism may hold. Assembly of yeast wild-type and mutant tubulin (*40*, *41*) is also uncoupled from GTP hydrolysis. An extreme example of this was found in assembly of a mixture of wild-type tubulin and β-T107W tubulin, which consumed 90 GTPs/dimer, incorporated into microtubules. Microtubules assembled from wild-type tubulin were found to contain 248 tubulin−GDP−P_i subunits and 173 tubulin−GTP subunits. These values were increased to 3924 and 819, respectively, with a mutant tubulin that had a low steady-state GTPase rate (β-T143G) and to 413 and 187 with microtubules formed with a mixture of wild-type and tubulin mutant that had an abnormally high GTPase rate (β-T107W). What was puzzling was that although MTs formed with the mutant β-T107W tubulin contained more GTP and GDP−P_i, microtubule dynamic instability was enhanced compared to wild type. It was suggested (*41*) that the GTPase rate, as well as the rate at which tubulin subunits undergo a conformation change after adding to the microtubule end, influences microtubule dynamics.

Summary. It is concluded that under the conditions used for the [^{32}P]GTP pulse labeling (*31*) ^{32}P was incorporated into the entire microtubule from [^{32}P]P_i, rather than into a tubulin−GDP−P_i cap from [^{32}P]GTP. Thus, there is no evidence that tubulin−GDP−P_i subunits with nonexchangeable P_i accumulate in microtubules. We had previously shown that tubulin−GDP−P_i subunits with exchangeable P_i do not stabilize microtubules (*28*).

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