Microtubule Dynamic Instability Does Not Result from Stabilization of Microtubules by Tubulin-GDP-P_i Subunits†

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ABSTRACT: The proposal that microtubule dynamic instability results from stabilization of microtubule ends by tubulin-GDP-P_i subunits (where P_i is inorganic phosphate) [Melki et al. (1996) *Biochemistry 35*, 12038] was based on studies of GTP hydrolysis and microtubule assembly that showed that tubulin-GDP-P_i subunits can transiently accumulate at microtubule ends. There is no direct evidence that GDP-P_i-subunits can stabilize microtubules under conditions where dynamic instability is observed and this has been inferred from the observation that tubulin-GDP-BeF_n subunits stabilize microtubules. To test if tubulin-GDP-P_i stabilizes microtubules we sought evidence for a synergism between the effect of P_i and BeF_n. We found, however, that P_i antagonizes the effect of BeF_n by displacing it from tubulin subunits. The alternate mechanism in which P_i inhibits BeF_n stabilization of microtubules by displacing fluoride from beryllium was ruled out from the ^{9}Be and ^{19}F NMR spectra in the presence and absence of P_i. Further evidence that tubulin-GDP-BeF_n is not an analogue of tubulin-GDP-P_i and that tubulin-GDP-P_i is not responsible for maintaining the growth phase in microtubules manifesting dynamic instability was provided by our observation that P_i did not decrease the disassembly rate under conditions where tubulin-GDP-P_i subunits are expected to have formed. Results showing that BeF_n binds randomly to subunits in microtubules provided evidence that P_i dissociation from the tubulin-GDP-P_i intermediate formed during GTP hydrolysis occurs randomly rather than processively starting at the growing microtubule tip.

The two states that allow allosteric regulation of microtubule dynamics by guanine nucleotide are not yet defined. Tubulin-GDP subunits that dissociate rapidly constitute one state. Tubulin-GTP or tubulin-GDP-P_i[1] subunits presumably constitute the second state. Understanding the role of GTP in microtubules is important because as with other G-proteins, nucleotide hydrolysis and exchange are likely to be involved in regulation of function.

The presence of an extensive tubulin-GTP cap at microtubule ends was first indicated from the observation of a 7 min discrepancy between assembly of 40 μM tubulin into microtubules and formation of 40 μM P_i from GTP (*1*). The stabilizing effect of tubulin-GTP subunits was inferred from results with nonhydrolyzable GTP analogues, which form microtubules that are relatively stable to depolymerization by calcium and cold (*2*−*4*). However, evidence against a GTP cap came from a subsequent study showing coincidence between the GTPase and assembly rates (*5*); also, microtubules isolated 10−15 s after assembly did not contain measurable amounts of GTP (*6*). The GTP cap mechanism was, therefore, revised so that the cap is restricted to a monolayer of tubulin-GTP subunits (*7*−*12*).

An alternate model in which growing microtubules are stabilized by a cap of tubulin-GDP-P_i subunits was proposed from the finding that microtubules isolated on a filter about 10 s after initiating assembly contain P_i from GTP hydrolysis (*11*). Although these results were not confirmed (*6*), subsequent study using a continuous spectrophotometric assay of the release of P_i from assembling microtubules (*13*) showed that P_i would be lost from microtubules too rapidly to be detected with a filter assay in which the dead time was 10−15 s (*6*). The half-time for P_i release was about 6 s, whereas GTP in microtubules was largely hydrolyzed in about 3 s. Since P_i release is slower than GTP hydrolysis, tubulin-GDP-P_i subunits accumulate in growing microtubules.

It has not been possible to determine whether tubulin-GDP-P_i subunits can stabilize microtubule ends; this was inferred (*13*) from results with the fluoride adducts of beryllium [BeF_n, in which n is presumably 3 (*14*)] and aluminum. These P_i analogues bind stoichiometrically and competitively with P_i (*15*) to tubulin-GDP subunits in microtubules and reduce the subunit dissociation rate (*15*, *16*). The assumption that tubulin-GDP-BeF_n resembles tubulin-GDP-P_i is, however, unproven. In fact, X-ray analysis of the complex between myosin and MgADP-BeF_x showed that this resembles the myosin−ATP species before hydrolysis (*17*). A similar conclusion was derived for a G-protein in which the geometry of the transducin-GDP-AlF_4 complex resembles a pentavalent intermediate in GTP hydrolysis, rather than a transducin-GDP-P_i complex (*18*).

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[1] Abbreviations: BRB buffer, 80 mM sodium 1,4-piperazinediethanesulfonate (Na-Pipes), 1 mM $MgCl_2$, and 1 mM [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid (EGTA); P_i, inorganic phosphate; MT, microtubule.

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These structural studies suggest that tubulin-GDP-BeF_n is an analogue of tubulin-GTP rather than tubulin-GDP-P_i, so that results with BeF_n may not be valid for estimating the stabilization from a tubulin-GDP-P_i cap.

There are three additional reasons to question whether growing microtubules are stabilized by a tubulin-GDP-P_i cap. First, using video microscopy to study the dynamics of individual microtubules we and others (*19*, *20*) failed to observe stabilization of microtubules by high concentrations of P_i. Second, we have measured a standard free energy change equal to +0.7 kcal for the two-step reaction sequence in which GMPCPP in microtubules hydrolyzes and P_i is released (*21*):

$$\text{tubulin-GMPCPP-MT} \leftrightarrow \text{tubulin-GMPCP-}P_i\text{-MT} \leftrightarrow \text{tubulin-GMPCP-MT} + P_i \quad (1)$$

On the basis of the 25 mM K_d for dissociation of P_i from microtubules (*15*) ΔG is +2.18 kcal for P_i dissociation in the second step in eq 1. The free energy for the first step in eq 1 in which the triphosphate is hydrolyzed is, therefore, only −1.48 kcal ($\Delta G_{overall} = +0.7 = -1.48 + 2.18$). The small free energy for nucleoside triphosphate hydrolysis requires that the remaining 3.7 kcal of energy from the triphosphate bond (5.18 kcal − 1.48 kcal; *20*) be stored in the microtubule lattice. The energy stored in tubulin-GMPCP-P_i subunits, and presumably also in tubulin-GDP-P_i subunits, is likely to be manifested as an enhanced dissociation rate so that these subunits would not be stabilizing, as suggested (*11*, *13*, *15*, *16*). A third reason for questioning whether microtubules are stabilized by a tubulin-GDP-P_i cap comes from an insightful analysis of the low affinity of P_i for microtubules (*22*). It was pointed out that "... if release of P_i increases the instability of microtubule ends, this would require a correspondingly less favorable dissociation of P_i." The low affinity of P_i (*15*), therefore, controverts stabilization of microtubules by GDP-P_i subunits.

We have again focused on determining the effect of P_i on microtubules and have used the P_i analogue BeF_n to determine whether failure to observe an effect of P_i on dynamics (*19*, *20*) resulted from an unmet requirement for multiple contiguous tubulin-GDP-P_i subunits to generate a stable microtubule end. Also, we tested a mechanism in which P_i binding is processive, starting with tubulin-GDP subunits that interface with a cap of tubulin-GTP (or tubulin-GDP-P_i) subunits at the microtubule end. Studies of the kinetics of shortening of BeF_n-treated microtubules showed that binding of BeF_n and presumably also P_i to microtubules is random rather than processive. Most important, we did not observe stabilization of microtubules by P_i under conditions where P_i is bound to tubulin-GDP subunits in the microtubule. P_i was found to reverse the BeF_n stabilization of microtubules, apparently by displacing BeF_n from tubulin-GDP-BeF_n subunits. The opposing effects of BeF_n and P_i indicates that tubulin-GDP-BeF_n is not an analogue of tubulin-GDP-P_i and we conclude that tubulin-GDP-P_i subunits are not responsible for stabilizing microtubule ends.

EXPERIMENTAL PROCEDURES

Beef brain tubulin was purified by two cycles of thermal-induced assembly and disassembly, followed by chromatography on phosphocellulose (*23*). The protein that did not bind to the phosphocellulose was concentrated with an Amicon YM10 membrane. Protein that denatured during the several hours required for the concentration step and/or during freezing, storage, and thawing was removed immediately before the tubulin was used by centrifugation this at 13800*g* (Microfuge) for 10 min at 4 °C. All reactions were in BRB buffer (80 mM $P_i$pes, pH 6.8, 1 mM EGTA, and 1 mM $MgCl_2$) at 37 °C.

Real-time measurements of microtubule dynamics were analyzed in reactions at 37° using video-enhanced DIC (differential interference contrast) microscopy. Microtubules were assembled at the ends of sea urchin axonemes (provided by E. D. Salmon) that bound to a cover slip in a flow cell (*24*) with an approximately 2.5 μL capacity. This small volume was replaced in 10−20 s by using filter paper to induce rapid flow of 20 μL of a displacing solution. Images from a Hamamatsu video camera were recorded on VHS tape and were analyzed with a computer-based system (*25*).

Tetracoordinated complexes containing beryllium, fluoride, and the OH moiety from water form rapidly in aqueous solutions (*26*), with the fluoride stoichiometry progressively increasing from 1 to 4 with increasing concentrations of fluoride (*9*). Thus, BeF_n was formed by including $BeSO_4$ and NaF in reaction mixtures. Studies of stabilization of microtubules by this substance were done in three stages. Microtubules were first assembled with 12−14 μM tubulin, 3 mM $MgCl_2$, and 1 mM GTP for 3−5 min to generate lengths that were sufficient to allow later study of their disassembly kinetics. Next, the microtubules were elongated for 10 min in the same mixture with 100 μM $BeSO_4$ and 25 mM NaF and either 33 mM Na_2SO_4, 50 mM P_i or 50 mM ethylphosphonate. The ionic strength is equal under these three conditions and from the dissociation constant for reaction of Mg with SO_4 (*27*) and with the phosphate dianion (*28*) the free Mg concentration is calculated to be 0.55 mM with SO_4 and 0.38 mM with P_i. During the 10 min reaction the portion of the microtubule that had formed before addition of BeF_n was viewed continuously to be certain that it was stable; meanwhile, most of the microtubules elongated beyond the field of view. Finally, microtubule disassembly was induced by addition of BRB buffer only or with the same solution used for the second phase of the reaction sequence, minus tubulin. Use of a diluent without BeF_n assured that the last portion of the microtubule that disassembled was exposed to BeF_n-free conditions for the longest time. The NaF concentration was 5 mM NaF in the experiments in Figures 2 and 3, since this condition formed microtubules that were sufficiently unstable that the rate of disassembly could be measured along their entire length. NaF at 5 mM was also used for reactions of BeF_n with microtubules in glycerol since we wished to reproduce earlier results (*15*, *16*).

An immunofluorescence assay was used to measure BeF_n stabilization of microtubules in glycerol. Microtubules assembled for 10 min with 23 μM tubulin in BRB buffer with and without 3.4 M glycerol were diluted 50-fold into a solution containing 50 mM MES, pH 6.8, 0.5 mM EGTA, 0.25 mM $MgCl_2$, 0.1 mM GTP, 3.4 M glycerol, 100 μM $BeSO_4$, and 5 mM NaF. At varying times aliquots were diluted 500-fold into 1% glutaraldehyde in BRB buffer, and microtubules were isolated on a cover slip placed on a layer of polymerized rubber caulking at the bottom of a tube that

was centrifuged at 28 500 rpm in a Beckman SW41 rotor. Microtubules were visualized from the signal from a fluorescein-labeled goat anti-mouse antibody (Capel) used to detect a mouse monoclonal antibody against α-tubulin (DM1-α, from Sigma). Images were captured with a CCD (charge coupling device) camera and microtubule lengths were measured with the digitizing program in an Ambis 10 image processor (Hamamatsu Corp.).

Nuclear magnetic resonance spectra were recorded at room temperature on a Bruker AMX500 spectrometer at 70.28 MHz, with D_2O as an internal lock. Spectra were accumulated for 30 min and chemical shifts are relative to $BeSO_4$.

RESULTS

It has been proposed (*13*) that the dissociation of P_i from tubulin-GDP-P_i subunits in microtubules occurs by a processive path in which the reaction occurs sequentially from adjacent subunits. It is possible to test this proposal by studying the mechanism for binding of the P_i analogue BeF_n. Since the principle of detailed balance requires that reactions follow an identical path in their forward and reverse directions (*29*), the same route is followed for dissociation and binding of P_i to tubulin-GDP subunits in microtubules. Thus, if BeF_n is an analogue of P_i and if its binding is processive, then so too is P_i dissociation. We were interested in determining whether a processive mechanism holds since it predicts that exposure of microtubules to P_i (and with growing microtubules to BeF_n; Figure 1) will form multiple contiguous tubulin-GDP-P_i subunits. We had found (*12*) that microtubule ends can be stabilized by 13−14 contiguous tubulin-GTP subunits; therefore, if P_i binding is processive, it is expected to be highly effective in stabilizing microtubules.

Evidence for processivity was sought by determining whether the stabilization that results from a brief exposure to BeF_n is localized to the region of the microtubule that grew most recently (Figure 1). Binding to this region is predicted for a processive mechanism since P_i and BeF_n is required to bind first to the site from which P_i was most recently lost, with subsequent binding to adjacent tubulin-GDP subunits. Since the most recently formed tubulin-GDP subunits are generated from newly added tubulin-GTP, the first site for binding of BeF_n is expected to be near the growing end of the microtubule. To test this model microtubules were briefly elongated in the presence of BeF_n and then diluted into tubulin-free buffer to induce disassembly. Evidence that BeF_n bound preferentially to the growing end was sought by looking for a reduced disassembly rate in this portion of the microtubule.

Processivity for Binding of BeF_n to Microtubules. Although microtubule stabilization by BeF_n has been carefully characterized in buffer with 3.4 M glycerol (*15*, *16*), we wished to study this phenomenon under conditions compatible with dynamic instability and, therefore, did not include glycerol in the reactions since this inhibits dynamic instability. We found in video microscopy studies that microtubules are unstable when diluted into 100 μM $BeSO_4$/5 mM NaF, which was surprising since 50 μM BeF_n had been found to stabilize almost instantly microtubules in glycerol buffers (*16*). A field viewed 60 s after dilution had only 9

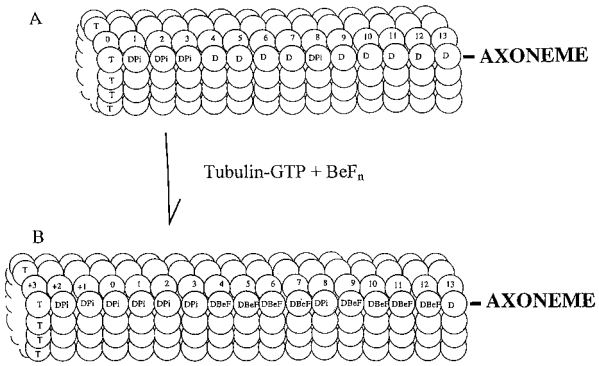

FIGURE 1: Implications of a processive mechanism for binding of BeF_n and dissociation of P_i. (A) Microtubules formed before addition of BeF_n are presumed to have a terminal monolayer of tubulin-GTP subunits (*12*) and a short cap of tubulin-GDP-P_i subunits (*13*) on a tubulin-GDP core. According to a strictly processive mechanism, P_i would be lost sequentially from subunits 3, 2, 1, 0, +1, etc. (B) Subsequent elongation of the microtubule shown in panel A in the presence of BeF_n first forms tubulin-GDP-BeF_n in subunit 4, since this is the subunit that had most recently lost P_i; subsequent binding occurs sequentially to subunits 5, 6, 7, etc. P_i that would ordinarily be lost sequentially from subunits 3, 2, and 1 during addition of tubulin-GTP subunits (+1, +2, +3, etc.) is retained, since addition of BeF_n to subunit 4 prevents loss of P_i from subunit 3 (and 2 and 1, etc.) Thus, subunits added in the presence of BeF_n will contain P_i, and subunits present before BeF_n will be progressively filled with this substance. P_i is shown in subunit 8 both before and after elongation with BeF_n because P_i dissociation is not processive (see below); the concentration of tubulin-GDP-P_i subunits is expected to progressively decrease toward the axoneme because these have had more time for dissociation to occur. Assuming that tubulin-GDP-P_i subunits destabilize adjacent tubulin-GDP-BeF_n subunits (see below), their relative absence in the "older" region of the microtubule would account for the greater stability of the portion of the microtubule near the axoneme (see Figures 2−4).

microtubules that had not fully disassembled (37 μm cumulative length) from 97 initial microtubules (456 μm cumulative length); equivalent results were observed in other viewing fields. Although the rate constant for BeF_n binding to microtubules is only 90 M^{-1} s^{-1} (*16*), only 4 s in 100 μM BeF_n would be required to incorporate this into 4% of the tubulin-GDP subunits. This amount of substitution would be expected to increase microtubule stability since the effect of BeF_n was found (*16*) to be described by

$$1/k_{obs} = f_-/k_- + f_+/k \qquad (2)$$

f_+ and f_- are the fraction of subunits with and without BeF_n and k_+ and k_- are the rates of dissociation of subunits with and without BeF_n. With k_-/k_+ equal to 25 (*16*), the rate will be reduced 2-fold when only 4% of subunits contain BeF_n.

To determine whether the discrepancy from the earlier results came from the absence of glycerol in the BeF_n diluent, microtubules assembled with or without 3.4 M glycerol were diluted 50-fold into glycerol-containing buffer with and without 100 μM BeF_n. Microtubules were unstable in glycerol buffer without BeF_n. For example, microtubules assembled with glycerol had a mean length of 1−2 μm and these were fully disassembled in 1 min; microtubules formed without glycerol were approximately 10-fold longer and their number concentration was reduced from an initial 22.4 microtubules/field ($n = 11$, SD 5.5) to 18.9 (11, 5.8) after 2 min, 15.1 (9, 2.8) after 5 min, and 3.9 (21, 2.2) after 20

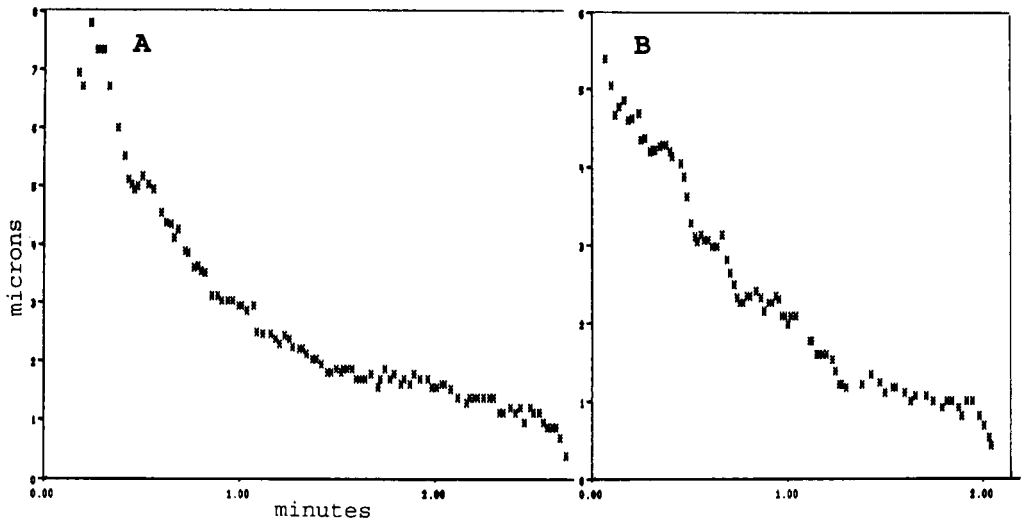

FIGURE 2: Attempt to detect processivity for binding of BeF_n to microtubules. Microtubules assembled at the end of axonemes with 12.7 μM tubulin were reacted for approximately 30 s with 12.7 μM tubulin subunits in 25 mM NaF and 100 μM $BeSO_4$; disassembly was induced by addition of the same solution, minus tubulin. In panel A the rate during disassembly of the first half of the microtubule was 200 s^{-1}; the rate was 40 s^{-1} for the other half. In panel B these rates were 107 and 41 s^{-1}, respectively.

min. In contrast, with microtubules assembled both with and without glycerol the number concentration was approximately the same at 1, 5, and 15 min after dilution into glycerol buffer with 100 μM BeF_n. Thus we confirmed the earlier finding (*16*) that microtubules in glycerol buffer are rapidly stabilized by BeF_n and deduce that BeF_n interacts with microtubules differently in the presence and absence of glycerol.

The lesser effect of BeF_n in the absence of glycerol may result because binding is weaker, so that the fraction of subunits as tubulin-GDP-BeF_n (i.e., f_+ in eq 2) is lower, or because BeF_n has a lesser effect on subunit dissociation (i.e., increased k_-). Evidence that weaker binding of BeF_n is responsible for the lesser effect in the absence of glycerol was obtained from studies in which assembled microtubules were elongated for 10 min in the presence of 100 μM BeF_n/5 mM NaF and then diluted to induce disassembly. The portion of the microtubule that was exposed to BeF_n for the entire 10 min disassembled at a rate equal to 72 s^{-1}, compared to 621 s^{-1} in a control (Table 1). Assuming a similar mechanism for microtubule stabilization by BeF_n in the presence and absence of glycerol, according to eq 2 the 9-fold reduction in rate with 100 μM BeF_n corresponds to 12.5% saturation of tubulin-GDP sites ($f_+ = 0.125$). This predicts a K_d equal to 700 μM; a 10−13 μM K_d was determined in glycerol buffer (*16*).

Having found that microtubules can be stabilized by 100 μM $BeSO_4$, this concentration was used to determine whether the stabilization induced by a brief (30 s) exposure of microtubules to BeF_n is localized to the region near the growing end. BeF_n was formed from $BeSO_4$ with 25 rather than 5 mM NaF since the lower concentration did not induce microtubule stabilization in 30 s. The requirement for a higher NaF concentration was surprising since the mole fraction of beryllium as BeF_3, which is presumably the reactive species for stabilizing tubulin-GDP subunits (*14*), only changes from 0.66 with 5 mM NaF to 0.61 with 25 mM F); however, the higher NaF increases the mole fraction as BeF_4 from 0.08 to 0.34 (*30*). The requirement for higher NaF for rapid stabilization of microtubules suggests that in the absence of glycerol microtubules are stabilized by BeF_4 rather than by BeF_3.

Table 1: Rate of Disassembly of Microtubules in BeF_n[a]

addition	rate (s^{-1}) (*n*, SD)
none	621 (8, 217)
BeF_n/5 mM NaF	72 (10, 58)
P_i	684[b]
Na_2SO_4	479 (6, 105)
BeF_n/25 mM NaF/Na_2SO_4	11(10, 8)
BeF_n/25 mM NaF/P_i	248 (13, 174)
BeF_n /25 mM NaF/ethylphosphonate	14 (8, 17)

[a] Microtubules that had been assembled at the ends of axonemes with approximately 15 μM tubulin in BRB buffer were treated for 10 min with a mixture of tubulin, 100 mM $BeSO_4$, and 25 mM NaF and either 33 mM $NaSO_4$, 50 mM P_i, or 50 mM ethylphosphonate; control reactions lacked the $BeSO_4$ and NaF. Disassembly was induced by addition of the same solution used for the BeF_n treatment but without tubulin. [b] Very rapid disassembly prevented tracing the length of individual microtubules and the rate was estimated from the reduction in length of 5 microtubules with a total length of 41 μm to 3.6 um in 18 s. The calculated rate is a lower limit for the reaction since 21 other microtubules with comparable length were fully disassembled when thefield was first observed.

The pattern for microtubule stabilization following a 30 s exposure to BeF_n was not in accord with a mechanism in which BeF_n binding is required to start near the growing end (Figure 1). Instead, the growing end was least stabilized so that the rate of disassembly progressively decreased as the microtubule disassembled (Figure 2). The ratio of the rate of disassembly of the first and second half of the microtubule was 3.2 ($n = 7$, SD 1.7). This is an approximate value since the decrease in the disassembly rate was progressive, so that a least-squares analysis of the data minimized the difference between the rate for the different portions of the microtubule.

It was possible that the progressive change in the disassembly rate after the 30 s exposure to BeF_n resulted because the portion of the microtubule nearest the axoneme became relatively enriched with tubulin-GDP-BeF_n subunits during the time that the microtubules were disassembling in tubulin-free buffer/BeF_n. This possibility was eliminated in an

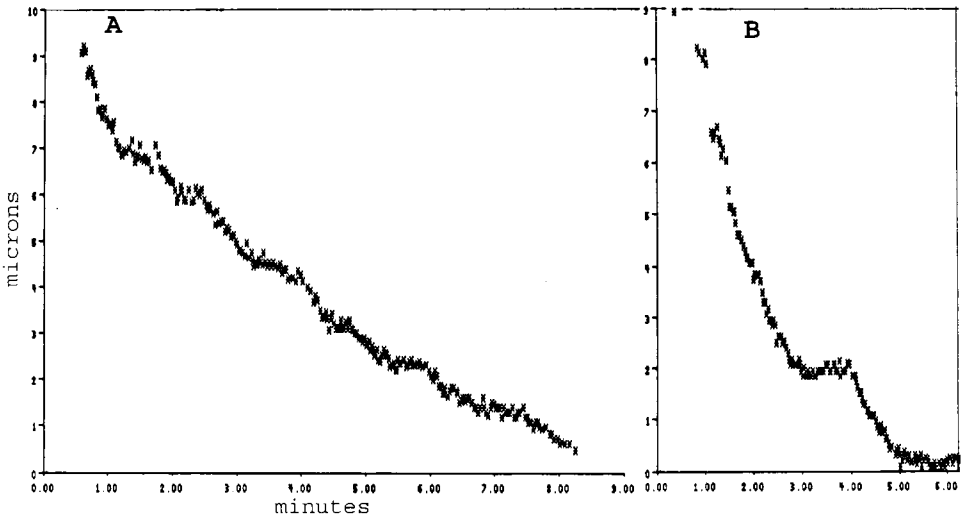

FIGURE 3: Disassembly of microtubules after reaction with BeF_n for 10 min. Kinetics are for the disassembly of microtubules that had formed at opposite ends of an axoneme in a reaction of 14 μM tubulin without BeF_n for about 4 min and for 10 min in the presence of 100 μM beryllium and 5 mM NaF. For the reaction in panel A, the microtubule length was 6.4 μm before addition of BeF_n (therefore, this portion of the microtubule was in BeF_n for the entire 10 min) and >14.7 μm after the 10 min reaction in the presence of BeF_n. After dilution (zero time in the kinetic plot), about 6 μm was lost during the approximately 15 s before the microtubule could be viewed. The rate of disassembly of the first and second half of the 6.4 μm length that had been in BeF_n was 33 and 20 s^{-1}, respectively. The microtubule described in panel B was 9 μm long before dilution, of which 5 μm had formed before addition of BeF_n; the disassembly rates were 84 and 33 s^{-1} before and after the pause at approximately 3 min.

experiment in which microtubules that had formed in the absence of BeF_n were further elongated for 10 min with a tubulin/BeF_n mixture, after which they were diluted into buffer without BeF_n or tubulin. NaF at 5 mM was used since this generated microtubules that were sufficiently unstable that it was possible to measure the rate of disassembly along their entire length. The predictions of a strictly processive mechanism for P_i dissociation are that maximum stabilization will be observed in the portion of the microtubule that formed in the presence of BeF_n. That is, if P_i dissociation is required to start at the interface of the GDP-P_i cap and GDP core, then binding of BeF_n to subunits at this interface would lock P_i into tubulin-GDP-P_i subunits in the portion of the microtubule that forms during assembly in the presence of BeF_n (Figure 1). The kinetics for dilution-induced disassembly did not support a processive mechanism since the portion of the microtubule that assembled in the presence of BeF_n was less rather than more stable than the portion that formed before addition of BeF_n (Figure 3). With almost all of the microtubules, the portion that formed in the presence of BeF_n was fully disassembled in the 15−30 s required for dilution and refocusing (Figure 3A). The low stability of the microtubule formed in the presence of BeF_n is not consistent with a processive mechanism in which binding is required to start at the interface of a tubulin-GDP-P_i cap and a tubulin-GDP microtubule core (Figure 1). Also, as was observed with microtubules that had been exposed to BeF_n for 30 s (Figure 2), a 10 min exposure resulted in preferential stabilization of the microtubule that was furthest from the growing microtubule end. The half of the microtubule that was distant from the axoneme end disassembled 2.6-fold faster than the half near the axoneme ($n = 12$, SD 1.4). It is noted that the greater stabilization was in the portion of the microtubule that had the most time for loss of BeF_n during the disassembly process.

It was postulated that the slower disassembly of the portion of the microtubule nearest the axoneme (Figure 3) resulted because this had a structure in which the A and B tubules of the doublet were both elongated, whereas only the A tubule was elongated in the more distal portion of the microtubule. This mechanism was tested by using ethylene glycol bis-(succinimidylsuccinate)- (EGS-) cross-linked single microtubules as seeds for elongating microtubules in the presence of BeF_n. Again, the rate was slower as the disassembly progressed toward the seed (Figure 4), ruling out the postulated mechanism: the rate for the half of the microtubule remote from the axoneme was 2.7 times ($n = 10$, SD 1.4) faster than the half near the axoneme.

Can Microtubules Be Stabilized by P_i? Microtubules assembled on axonemes without P_i were elongated for 10 min with tubulin in 50 mM P_i. When disassembly was induced by dilution into buffer containing P_i, neither the portion of the microtubule formed in the absence nor in the presence of P_i was unusually stable; most microtubules had fully disassembled in the 15−25 s required for addition of the diluent and refocusing on the field. The microtubules that could be observed to disassemble did so at a rate equal to 684 s^{-1} (Table 1), which is equivalent to that in a control reaction in which an identical ionic strength was maintained with Na_2SO_4 (Table 1).

Since P_i binding is not processive so as to form multiple contiguous tubulin-GDP-P_i subunits, a different approach was use to facilitate their formation. Microtubules were reacted with a mixture of BeF_n and P_i. Under these conditions the probability for forming n contiguous tubulin-GDP subunits containing either P_i or BeF_n is equal to the nth power of the sum of the mole fraction of subunits with BeF_n and the mole fraction of subunits with P_i. It was expected that binding of P_i would enhance the effect of BeF_n in regions of the microtubule in which the number of contiguous tubulin-GDP-BeF_n is insufficient to stabilize maximally the microtubule end. We found, however, that P_i inhibited rather than enhanced the stabilization induced by BeF_n (Table 1, Figure 5). Representative results showing the dramatic reversal by

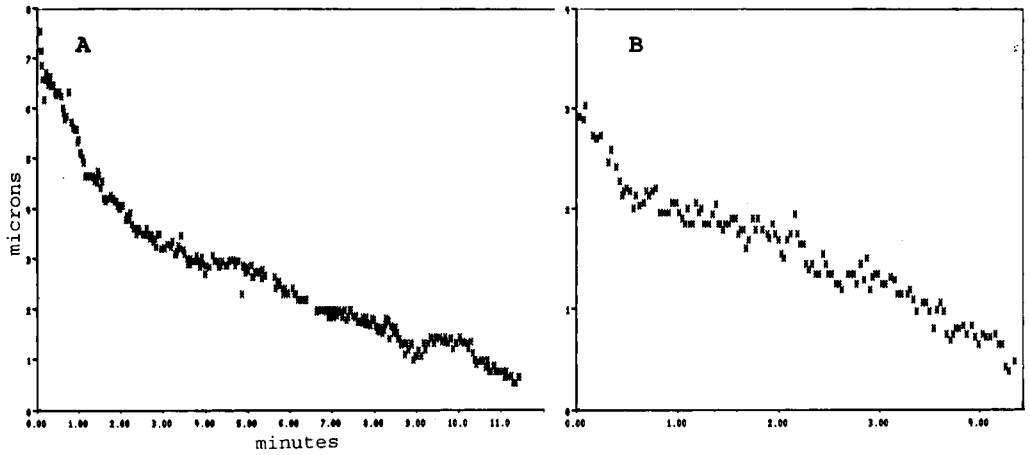

FIGURE 4: Effect of BeF_n on the stability of microtubules that had been assembled at the ends of EGS seeds. Microtubules assembled without BeF_n were elongated for an additional 10 min in 100 μM beryllium and 5 mM NaF. For panel A the length was 8.2 μm before dilution with BRB buffer, 4.1 μm of which had been formed before addition of beryllium fluoride; in panel B the corresponding lengths were 4.5 and 2.2 μm, respectively. The rates in panel A started at 53 s^{-1} and progressively slowed to 8 s^{-1} after 6 min. In panel B the rates ranged from 53 to 12 s^{-1}. Zero time in both reactions corresponds to 20−30 s after dilution with buffer.

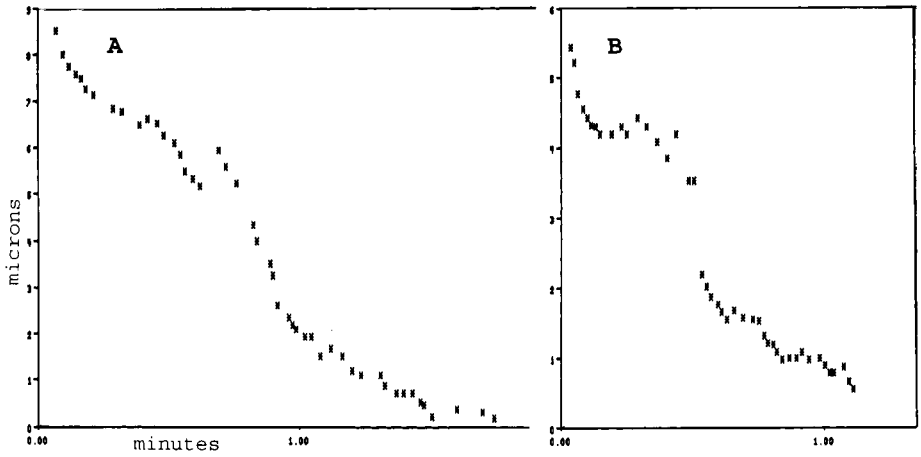

FIGURE 5: Effect of P_i on BeF_n stabilization of microtubules. Microtubules assembled without BeF_n were further elongated for 10 min in 25 mM NaF/100 μM $BeSO_4$ and 50 mM P_i and then diluted with the same solution, minus tubulin. The predilution length of the microtubule described in panel A was 11.3 μm, of which 3.5 μm had formed before addition of BeF_n. The rate during the first 0.7 min was 111 s^{-1} and this increased to 294 s^{-1} for about 0.3 min and then decreased to 84 s^{-1}. The tip of the microtubule described in panel B was out the range of view for 45 s after dilution with buffer; the rate was 300 and 452 s^{-1} before and after a brief pause.

P_i of the microtubules stabilization by BeF_n are shown in Figure 6. The P_i analogue ethylphosphonate did not similarly reverse the stabilization of microtubules by BeF_n (Table 1).

The possibility that P_i inhibits the stabilization of microtubules by displacing fluorine from BeF_n was ruled out from the ^{9}Be NMR spectrum in the presence and absence of P_i. The fraction of beryllium with 1−4 coordinated fluorides can be determined from the NMR spectrum (*31*) and the high sensitivity of this method is illustrated from our results with 2.5 and 5 mM NaF. It was possible (Figure 7) to detect the change in the percent BeF_n (n = 2, 3, and 4) from 35, 53, and 3.5 with 2.5 mM NaF to 21, 66, and 9 with 5 mM NaF (*30*). Results from the ^{9}Be NMR analysis were confirmed with ^{19}F NMR. In BRB buffer (pD 6.8) containing 1 mM $BeSO_4$ and 25 mM NaF, the integrated intensity of the signals (normalized relative to the signal from NaF) from BeF_3 and BeF_4 were 1.28 and 0.58, respectively, in the absence of P_i. In the presence of an equimolar mixture of 25 mM Na_2HPO_4 and NaH_2PO_4 the relative intensities were 1.08 and 0.57, respectively. Identical spectra were observed when these were recorded with a 1 or 5 s relaxation delay.

DISCUSSION

There is no direct evidence that microtubules can be stabilized by a cap of tubulin-GDP-P_i subunits. Instead, slow dissociation of tubulin-GDP-P_i has been inferred from studies with BeF_n (*11*, *16*), which forms tubulin-GDP-BeF_n in microtubules and decreases the rate of subunit dissociation about 25-fold compared to tubulin-GDP. The conclusion that tubulin-GDP-P_i stabilizes microtubules is, however, not in accord with studies of the dynamics of individual microtubules (*19*, *20*), showing that high concentrations of P_i did not decrease the rate of subunit dissociation or the catastrophe frequency.

On the basis of results obtained in glycerol buffer, it would appear that microtubules contained tubulin-GDP-P_i subunits under the conditions where P_i was found not to stabilize microtubules (Table 1; *19*). A rate constant for P_i binding equal to 5 M^{-1} s^{-1} is calculated from the ratio of the association and dissociation rate constants (equal to the K_d), with K_d equal to 25 mM (*16*) and the dissociation rate constant equal to 0.126 s^{-1} (*13*). Thus, in reactions with 0.05 M P_i the expected half-time for binding is 2.8 s; a 10

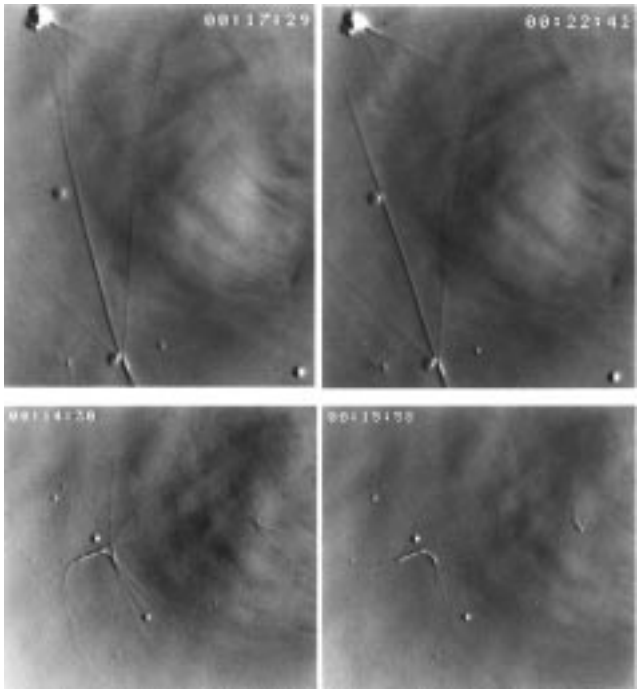

FIGURE 6: Dilution-induced disassembly of the microtubules that had been reacted with BeF_n in the presence (top) and absence (bottom) of P_i; conditions were as described for Figure 5. Dilution was induced at 15:00 min for the reaction with P_i and at 14:05 min for the reaction without P_i.

min incubation with P_i was used in our studies. The lack of stabilization by P_i indicates that in the absence of glycerol the 25 mM K_d for P_i and/or the 0.126 s^{-1} rate constant for P_i dissociation does not hold, and/or that microtubules are not stabilized by tubulin-GDP-P_i subunits.

The proposed processive path for GTP hydrolysis and P_i dissociation (*13*) would be expected to enhance the stabilizing effect of added P_i, if it were true that tubulin-GDP-P_i subunits can stabilize microtubule ends. According to a processive mechanism for GTP hydrolysis, this occurs predominantly at the interface of a GTP cap and the microtubule's GDP-core (*32*−*34*). The dissociation (and binding) of P_i would be similarly processive, starting at the interface of the tubulin-GTP cap and proceeding into adjacent tubulin-GDP subunits (Figure 1). This path would generate multiple contiguous tubulin-GDP-P_i subunits and these are expected to have an enhanced stabilizing effect on microtubule ends since it was found (*16*) that the reduction in the subunit dissociation rate is proportional to more than the first power of the mole fraction of microtubule subunits containing BeF_n. The probability of n contiguous tubulin-GDP-BeF_n subunits is proportional to the nth power of the mole fraction of tubulins containing BeF_n. Our finding (*12*) that 13 or 14 contiguous tubulin-GMPCPP subunits are necessary and sufficient to stabilize a microtubule end further supports our postulate that processive binding of P_i would be highly effective in stabilizing microtubules.

Binding of BeF_n and presumably P_i is not processive since the stabilization induced by exposure of microtubules to BeF_n was not greatest in the region of the growing end of the microtubule (Figures 2−4). Instead, maximal stabilization was in the region most distal to the growing end. Thus, the path for P_i binding is not expected to generate exclusively contiguous tubulin-GDP-P_i subunits. It is nevertheless possible to form multiple contiguous tubulin-GDP-P_i subunits, but as described above this is statistically likely only when microtubules contain a significant mole fraction of subunits with P_i.

The lack of microtubule stabilization by P_i did not result from failure to form sufficient contiguous tubulin-GDP-P_i subunits but instead resulted because the microtubule lattice is not stabilized by binding of P_i to tubulin-GDP subunits.

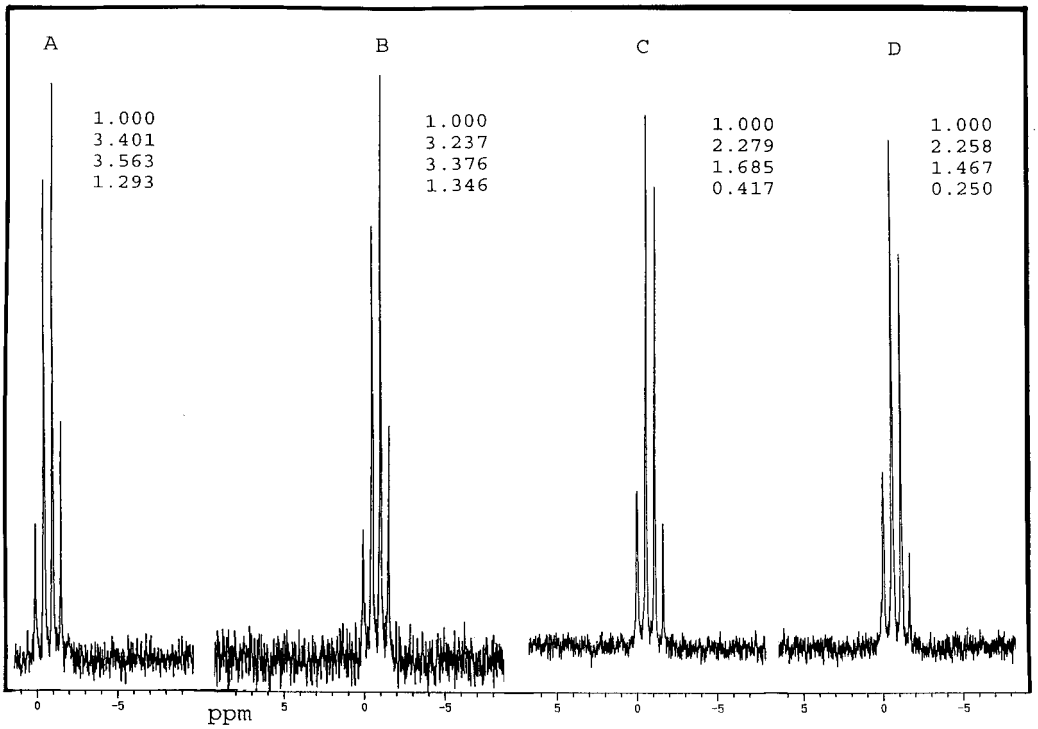

FIGURE 7: Nuclear magnetic resonance spectra of BeF_n in the presence and absence of P_i. All spectra are from 100 μM $BeSO_4$ in BRB buffer, with 25 mM NaF (A), 25 mM NaF and 50 mM P_i (B), 5 mM NaF (C), and 2.5 mM NaF (D).

This conclusion is derived from our observation that P_i and BeF_n did not act synergistically in stabilizing microtubules. Synergism would result if binding of both of these substances to GDP subunits stabilizes the microtubule lattice and if, as with GMPCPP (*12*), microtubule stabilization is enhanced by multiple contiguous stabilizing subunits. Synergism is predicted because the probability for forming *n* contiguous tubulin-GDP subunits containing either P_i or BeF_n is proportional to the *n*th power of the sum of the mole fraction of tubulin-GDP subunits containing either P_i or BeF_n. In contrast with this prediction, P_i antagonized rather than enhanced BeF_n stabilization of microtubules (Figures 5 and 6). Destabilization by P_i resulted because it displaced BeF_n from the tubulin-GDP subunits and because tubulin-GDP-P_i subunits are unstable in the microtubule lattice. Finally, our finding that P_i reversed the effect of BeF_n requires that P_i is bound to microtubules when this substance is present; greater binding of P_i would occur when BeF_n is not present to compete for tubulin-GDP subunits. Therefore, our finding that P_i did not increase the stability of microtubules in the absence of BeF_n means that tubulin-GDP-P_i subunits do not stabilize microtubule ends.

Evidence that binding of P_i further decreases the stability of tubulin-GDP subunits comes from earlier results showing that P_i dramatically increased the catastrophe and disassembly rate and decreased the rescue rate with microtubules formed with chick erythrocyte and pig brain tubulin (*20*). The greater effect of P_i seen with erythrocyte microtubules (*20*) suggests that erythrocyte-specific β-6/α-1 tubulin (*35*) has enhanced affinity for P_i. Our observation that tubulin-GDP-P_i subunits destabilize the microtubule lattice provides evidence that the increased dynamics seen with this substance (*20*) did not result from a medium effect but instead resulted from P_i binding to the microtubule.

Our finding that the P_i analogue ethylphosphonate did not inhibit BeF_n-induced microtubule stabilization (Table 1) provides evidence against a mechanism in which P_i reduces microtubule stabilization by BeF_n by displacing fluoride from beryllium, to reduce the concentration of BeF_n. If P_i acted by reacting with beryllium then the greater basicity of ethylphosphonate compared to P_i would be expected to make it more reactive with beryllium and thus more effective in inhibiting the effect of BeF_n. Also, the monodentate complex of pyrophosphate and beryllium was completely displaced when the fluoride concentration was only 20 mM (*26*); therefore, it is not expected that P_i would be bound to beryllium in the presence of the 25 mM fluoride used in our experiments. Finally, ^9Be and ^{19}F NMR analysis of BeF_n in the presence and absence of P_i showed that the inhibitory effect of P_i on BeF_n stabilization did not result from its displacing fluoride from beryllium (Figure 7).

We do not have an unambiguous interpretation of the progressive decrease in the rate of disassembly of microtubules exposed to BeF_n (Figures 2−4). It was previously found that the disassembly rate can vary along the microtubule length (*36*−*38*), but this variability was random. The greater stabilization near the nucleating axoneme or EGS seed did not result because of longer exposure to BeF_n. Rather, because the dilution was into BeF_n-free buffer, the last-disassembling portion of the microtubule had the most time for dissociation of BeF_n. It is suggested that the greater stabilization of the portion of the microtubule near the seed results because this first-formed portion of the microtubule has had more time to release P_i from tubulin-GDP-P_i subunits (Figure 1). If tubulin-GDP-P_i subunits can vitiate the stabilizing effect of adjacent tubulin-GDP-BeF_n subunits, the microtubule will be most stable where the probability is greatest that P_i has been lost. The very low stability of the portion of the microtubule that forms in the presence of BeF_n may result because this region is enriched with tubulin-GDP-P_i subunits, with dissociation of P_i inhibited because of binding of BeF_n to subunits that have lost P_i.

In summary, we have obtained evidence that tubulin-GDP-P_i subunits destabilize the microtubule lattice so that their presence at microtubule ends cannot be responsible for microtubule dynamic instability as previously proposed (*12*, *15*). Our results indicate that tubulin-GDP-BeF_n is an analogue of tubulin-GTP rather than of tubulin-GDP-P_i. This conclusion is supported by results with G-proteins and myosin where BeF_n and bind to the site normally occupied by the γ-phosphate moiety of GTP and ATP (*39*−*41*) and mimics the effect of the nucleoside triphosphate. The lack of microtubule stabilization by tubulin-GDP-P_i and the failure to observe a large tubulin-GTP cap suggests that growing microtubules are stabilized by a monolayer of tubulin-GTP subunits (*12*).

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