Stabilization of Microtubules by Tubulin–GDP–P_i Subunits†

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ABSTRACT: Microtubule dynamic instability has been accounted for by assuming that tubulin subunits at microtubule ends differ from the tubulin–GDP subunits that constitute the bulk of the microtubule. It has been suggested that this heterogeneity results because ends contain tubulin subunits that have not yet hydrolyzed an associated GTP molecule. Alternatively, in a recent model it was proposed that ends contain tubulin–GDP–P_i subunits from which P_i has not yet dissociated. The models differ in their predicted response to added ligands: because GDP in subunits in microtubules does not exchange with nucleotide in solution, the heterogeneity from a tubulin–GTP cap will not be eliminated by added GTP; however, the dissociability of P_i in tubulin–GDP–P_i subunits will allow a heterogeneity resulting from a tubulin–GDP–P_i cap to be eliminated by added excess P_i. Elimination of the heterogeneity is expected to be manifested by an elimination of dynamic instability behavior. Using video microscopy to study the kinetic behavior of individual microtubules under reaction conditions where dynamic instability is the dominant mechanism for microtubule length changes, we have determined the effects of 0.167 M P_i on the rate of subunit addition in the elongation phase, the rate of subunit dissociation in the rapid shortening phase, and the rates of the phase transitions from elongation to rapid shortening and from rapid shortening to growing. Since 0.167 M P_i did not decrease the subunit dissociation rate in the rapid shortening phase or the rate of the phase transition from growing to rapid shortening, our results provide no support for the hypothesis that tubulin–GDP–P_i subunits are responsible for dynamic instability behavior of microtubules. Absence of P_i-mediated stabilization of microtubules was also indicated by the observations that P_i did not reduce the tubulin subunit concentration required to maintain a steady state or decrease the rate of dilution-induced microtubule disassembly in the presence or absence of 2 M glycerol.

Individual microtubules assembled in vitro undergo rapid elongation and shortening under conditions where the total microtubule mass remains constant. The surprising thing about this steady state is that elongating and rapidly shortening microtubules are in separate populations which interconvert infrequently. This behavior, which was implicated from studies of the change in microtubule number and length under steady-state conditions (Mitchison & Kirschner, 1984a,b), has been observed directly by using real-time video microscopy (Cassameris et al., 1988; Horio & Hotani, 1986; Sammak & Borisy, 1988; Schulze & Kirschner, 1988; Walker et al., 1988).

It is not known how the kinetically distinct microtubule populations differ chemically. One possibility is that elongating microtubules are stabilized by a cap of recently added tubulin–GTP subunits, which dissociate from ends very slowly (Kirschner & Mitchison, 1986). Such stabilization of any one microtubule is expected to be transient, since GTP that is incorporated into microtubules with tubulin subunits is eventually hydrolyzed. When tubulin–GDP subunits are at microtubule ends, rapid shortening ensues and the subunits released elongate the remaining microtubules. Although the GTP cap model can account for microtubule dynamics, there is no direct evidence that GTP is contained in microtubules that exhibit dynamic instability behavior.

The GTP cap model is especially attractive because it links the role of GTP hydrolysis in microtubule assembly with that in the many G-proteins that regulate cellular processes (Allende, 1988). G-Proteins associate with macromolecular structures when they have bound GTP and dissociate when the GTP molecule is hydrolyzed (Allende, 1988). The parallel between the behavior of G-proteins and tubulin motivated a recent study of the effect of fluorine-containing phosphate analogues on microtubule dynamics (Carlier et al., 1988). It is known that compounds such as AlF_3^- bind to G-proteins at the site normally occupied by the γ-phosphate of GTP to form a stable ternary complex with GDP (Bigay et al., 1987; Sternweis & Gilman, 1982). The behavior of this ternary complex resembles that of the complex containing GTP. Although it has been found that combinations of GDP and fluoroaluminate species are incapable of activating tubulin for polymerization (Humphreys & MacDonald, 1988), formation of a complex in microtubules consisting of tubulin, GDP, and BeF_3^- with a unitary stoichiometric ratio has been demonstrated (Carlier et al., 1988). Furthermore, it was reported that the rate of disassembly was significantly decreased by P_i[1] following a 15-fold dilution of steady-state microtubules into buffer containing 50% sucrose.[2] This stabilization by P_i was interpreted (Carlier et al., 1988) as indicating that P_i binds to tubulin–GDP subunits in microtubules and reduces the dissociation rate. With steady-state microtubules, formation of slowly dissociating GDP–P_i subunits after GTP hydrolysis was presumed to prevent depolymerization of the microtubule GDP core. To test this interesting proposal, we have directly measured the effect of P_i on the rate of subunit dissociation from microtubules undergoing rapid shortening and the frequency at which microtubules undergo transition from a

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[1] Abbreviations: DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAPS, microtubule-associated proteins; MES, 4-morpholineethanesulfonic acid; P_i, inorganic phosphate.

[2] That the diluent contained both 3.4 M glycerol and 50% sucrose was in error (personal communication from M. F. Carlier).

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regimen of slow growth to a regimen of rapid disassembly. We have also determined the effect of P_i on the steady-state critical subunit concentration, under conditions where microtubules demonstrate dynamic instability behavior.

Materials and Methods

The protein used in video microscopy studies was porcine brain tubulin purified by two cycles of assembly and disassembly in a buffer of 100 mM MES, 1 mM EGTA, 0.5 mM $MgSO_4$, and 3.4 M glycerol, pH 6.6, followed by passage over phosphocellulose and a third cycle of assembly in 1 M sodium glutamate, as described previously (Voter & Erickson, 1984). Bovine brain tubulin (Zeeberg et al., 1980), purified by phosphocellulose chromatography (Weingarten et al., 1975), was used for studies other than those involving video microscopy. Flagellar axonemes were prepared from *Lytechinus pictus* according to the method of Bell et al. (1982).

Reactions were at 37 °C unless stated otherwise. Rates of dilution-induced disassembly were measured as described elsewhere (Caplow et al., 1985). The steady-state subunit concentration was determined in 1 mM Mg^{2+}, in the presence and absence of 0.167 M P_i, or in 0.111 M SO_4. Microtubules were assembled to steady state with [^{3}H]GTP and a GTP-regenerating system (Caplow et al. 1985), P_i or SO_4 was added from a concentrated stock solution, and reactions were diluted to 30–65 μM tubulin concentrations. After a 15-min incubation, which was found to be sufficient to attain a new, stable steady state, the solutions were centrifuged in an airfuge (at 37 °C), and the radioactivity in the pellet was measured. The specific activity of the guanine nucleotide was determined by measuring the reduction of radioactive nucleotide incorporated into polymer, produced by adding an amount of GTP equal to the tubulin concentration. The reduction is proportional to the ratio of the endogenous nucleotide to the sum of the added and endogenous nucleotide. Endogenous nucleotide was not precisely equal to the protein concentration, since the tubulin contained guanine nucleotide that apparently was released from protein that had denatured during storage and was removed by a centrifugation of the defrosted protein.

Microtubule dynamics were assayed by video microscopy (Walker et al., 1988). Reaction mixtures containing 1 mM GTP and approximately 2.7×10^7 axonemes/mL were prepared at 0 °C and then incubated in a sealed slide-coverslip chamber at 23 °C. Microtubule elongation and rapid shortening rates were measured from video recordings, using a computer-based system that allowed a point cursor to track the end of a microtubule (Walker et al., 1988). Changes in microtubule length were plotted as a function of time, and the elongation rate was determined by least-squares regression analysis.

It was previously found that the frequency of transition from elongation to rapid shortening and from rapid shortening to elongation is independent of the time spent in a particular phase, indicating that such events are random processes (Walker et al., 1988). As a result, it is possible to calculate the rate constants for the phase transition by summing the time spent by all measured microtubules in a particular phase and dividing this into the number of observed phase transitions.

Results

Reaction Conditions. Walker and Salmon (unpublished results) have found that microtubules formed with 16 μM tubulin at the plus end of axonemes at 23 °C grow at a rate of 37 subunits/s, undergo transition from elongation to rapid shortening regimen ("catastrophe") with a frequency of 0.0045 s^{-1}, and convert from rapid shortening to elongation regimen

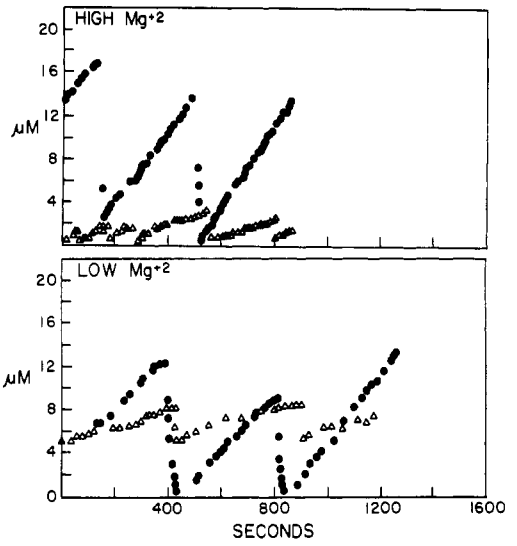


FIGURE 1: Video microscopy analysis of dynamics of a microtubule at an axoneme end. Representative length measurements from reactions 1 and 2 in Table I are shown here. The microtubule's plus and minus ends are described by a circle and a triangle, respectively.

("rescue") with very low frequency (0.0021 s^{-1}). As a result, during the 222-s mean lifetime (i.e., 1/0.0045) that microtubules spend in the elongation regimen they grow by about 5.1 μm, and because the mean lifetime in the shortening regimen is 476 s (i.e., 1/0.0021), this length is almost invariably completely lost from a microtubule that enters the rapid shortening regimen. The minus end grows 3.3 times more slowly, but catastrophes occur about 4 times less frequently, and consequently, here too, a relatively large excursion in length is observed when a catastrophe occurs. Large excursions in length allow accurate measurement of the effect of P_i on the parameters that characterize the dynamic instability phenomenon, and the conditions just described were, therefore, chosen for our studies.

Reactions were studied with either 0.167 M Na_2PO_4 or 0.111 M Na_2SO_4, which has the same ionic strength. This concentration exceeds the K_d for P_i dissociation from microtubules, which was estimated at 0.025 M (at 30 °C) (Carlier et al., 1988). Analysis of the effect of P_i on dynamic instability required the use of reaction conditions different from those used previously (Carlier et al., 1988). We could not use glycerol or sucrose buffers in our studies of the effect of P_i on dynamic instability since this phenomenon is not manifested in the presence of glycerol (Caplow et al., 1986) and is not expected to be observable in sucrose buffer, since the P_i-induced decrease in rate of disassembly (Carlier et al., 1988) would assure very small length changes during the time intervals when microtubules are in the rapid shortening regimen. We used an aqueous buffer with either 1 or 6 mM Mg^{2+}, since the dynamic instability phenomenon and microtubule dynamics have been extensively analyzed under these conditions (Walker et al., 1988; Caplow et al., 1988).

Control Reactions. Microtubules at axoneme ends were observed to undergo long periods of monotonic growth, interrupted by brief periods during which they underwent very rapid shortening (Figure 1). Rates were sensitive to the Mg^{2+} concentration. In reactions without added ligand (Table I, reactions 1 and 2), increasing Mg^{2+} from 1 to 6 mM resulted in an approximately 1.5-fold increase in the elongation rate and 1.5–3-fold increase in the rapid shortening rate at the two microtubule ends. MAP-free microtubules are extraordinarily reactive in the presence of high Mg^{2+}; the sum of the shortening rates at the plus and minus microtubule ends was about

Table I: Dynamic Properties of Microtubules in the Presence of P_i or SO_4 and at 23 °C

reaction	ligand[a]	tubulin concn[b] (μM)	end[c]	v_e[d] (SD) (μm/m)	n[e]	k_c[f] (s^{-1})	n[g]	v_{rs}[h] (SD) (μm/m)	n	k_r (s^{-1})	res:cat[i]
1	1 mM Mg^{2+}	16	+	1.4 (0.2)	10	0.0025	9	32 (30)	10	0.0046	1:10
			−	0.5 (0.1)	14	0.0018	10	44 (11)	10	0.22	10:10
2	6 mM Mg^{2+}	16	+	2.0 (0.5)	12	0.0030	11	52 (13)	8	<0.0068[j]	0:11
			−	0.8 (0.3)	20	0.0066	20	154 (29)	7	0.0029	1:20
3	1 mM Mg^{2+}	26	+	1.5 (0.1)	10	0.0036	11	27 (12)	11	0.025	5:11
	P_i		−	0.6 (0.2)	10	0.0036	10	106 (50)	8	0.31	4:10
4	1 mM Mg^{2+}	26	+	1.8 (0.3)	11	0.0029	10	44 (12)	9	<0.008[j]	0:10
	SO_4		−	0.7 (0.1)	16	0.0034	19	77 (20)	9	0.14	4:19
5	6 mM Mg^{2+}	26	+	1.4 (0.2)	8	0.0090	3	147 (53)	7	*k*	0:12
	P_i		−	0.6 (0.2)	14	0.0027	9	156 (63)	9		

[a] The P_i and Na_2SO_4 concentrations were 0.167 and 0.111 M, respectively, added to 100 mM Pipes, 1 mM EGTA, and 1 mM GTP. Results from one of several identical reactions are given here. [b] The subunit concentration remains constant, since there is exceedingly little net assembly in these reactions. [c] The more rapidly growing end was assumed to be the plus end. [d] Rate of net microtubule elongation. [e] Number of microtubules measured. In most cases reaction of the same axonemal microtubule may have been measured repeatedly, as it went through cycles of assembly and disassembly. This cannot be determined with certainty, since the axonemes were frequently bundled together so that our observation of regrowth of a microtubule with a similar orientation as one that just disappeared did not allow us to be certain that the same end was repeated elongated. [f] The frequency of conversion from the elongating to rapid shortening regimen was calculated from the number of transitions from growth to rapid shortening divided by the total time microtubule elongation was measured. [g] Number of catastrophe events observed. [h] In most cases the rate of rapid shortening was sufficiently rapid that it could best be determined by measuring the microtubule length before shortening commenced and the number of seconds until the microtubule fully disappeared. The error in these measurements is fairly large because disassembly was frequency complete in less than a second. [i] Ratio of the number of microtubules that revert from shortening to assembly regimen before complete disassembly to the number of catastrophes observes. [j] No rescues were observed, so that the rate constant reported here is an upper limit estimate. [k] k_r is not calculable since either zero or only one phase transition was observed.

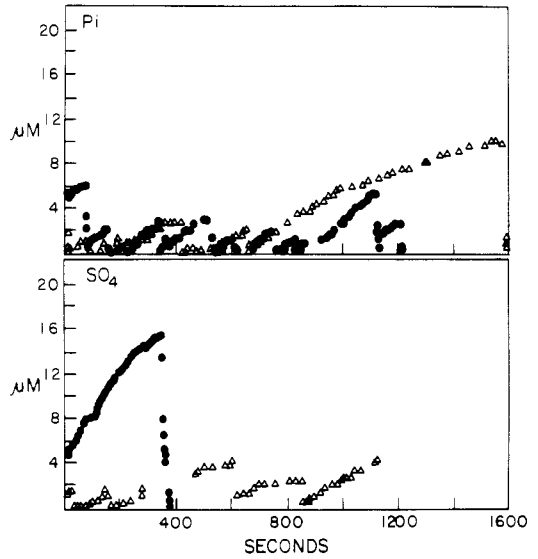


FIGURE 2: Effect of P_i on microtubule dynamics. Representative traces from reactions 3, 4, and 6 (Table I) are shown here.

200 μm/min (5500 subunits/s). This value agrees with a previous determination equal to 4500 subunits/s at 37 °C (Caplow et al., 1988), obtained with a radioisotope assay to follow microtubule disassembly in bulk populations after 1000-fold dilution into buffer containing 7.5 mM Mg^{2+}. The most dramatic effect of increasing Mg^{2+} from 1 to 6 mM was an approximately 75-fold reduction in the rescue rate (k_r) at the microtubule minus end; the plus end may be similarly affected, but because no rescue events were observed, we were only able to obtain an upper limit for k_r and this value was actually slightly higher than at low Mg^{2+}.

Effects of P_i. The most important observation in our studies with P_i was that this substance did not significantly influence the rate of subunit dissociation from microtubules in the rapid shortening regimen (cf. v_{rs} in reactions 3 and 4 in Table I and Figure 2). The effect of P_i on v_{rs} determined from microscopy was confirmed by using an isotope assay (Caplow et al., 1985) to measure microtubule shortening after a 1000-fold dilution. In the aqueous buffer used for microscopy the disassembly rates with P_i and SO_4 were virtually identical (Table II), and

Table II: Effect of P_i and SO_4 on Dilution-Induced Disassembly

diluent[a]	time (s)	% microtubule disassembled
control	10	28
	15	61
	20	67
P_i	5	34
	10	54
SO_4	5	22
	10	57

[a] Microtubules assembled with high specific activity [^{3}H]GTP were diluted 1000-fold into 100 mM Pipes, 1 mM EGTA, and 1 mM $MgCl^{2+}$, pH 6.8 (control), or into this buffer with either 0.167 M P_i or 0.111 M Na_2SO_4. Triplicate measurements were made at each time point.

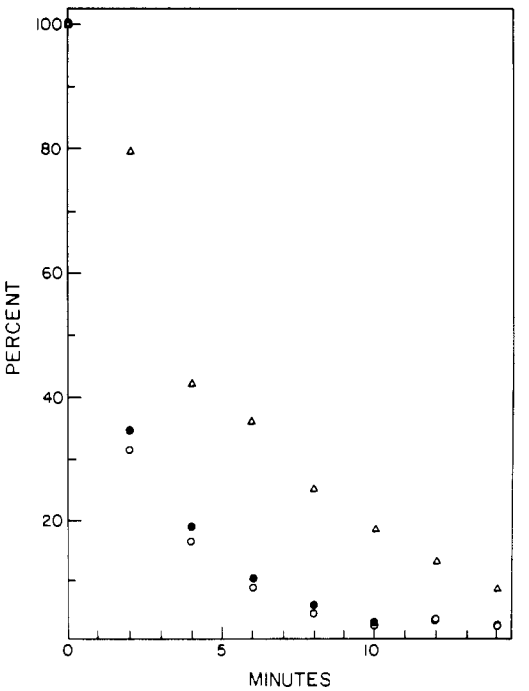


FIGURE 3: Effect of P_i on the rate of microtubule rapid shortening in the presence of 2 M glycerol. Microtubules assembled from 40 μM tubulin in the presence of [^{3}H]GTP were diluted 1000-fold into buffer (triangles) or into buffer containing 0.167 M P_i (solid circles) or 0.111 M Na_2SO_4 (open circles).

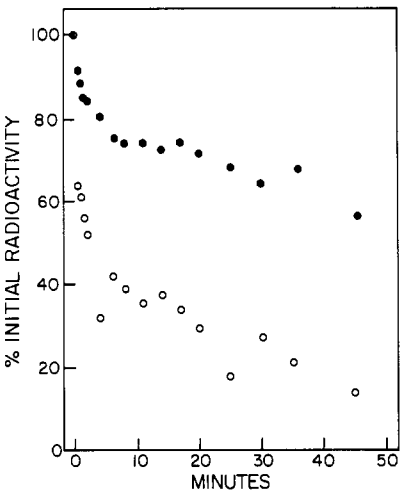


FIGURE 4: Effect of P_i on the rate of disassembly of microtubules in 50% sucrose. Microtubules were diluted 1000-fold into buffer containing either P_i (solid circles) or SO_4 (open circles).

this rate was about 1.5-fold greater than that in the absence of these substances. With 2 M glycerol the rapid shortening rates with P_i and SO_4 were again indistinguishable, and these were about 3-fold greater than that of a control (Figure 3). The microscopy assay had shown no significant difference between the shortening rates with P_i and SO_4 relative to a control (cf. sum of v_{rs} at the plus and minus ends in reaction 2 and reactions 3 and 4 in Table I); however, the relatively large variation in rate of rapid shortening between different microtubules seen in the microscopy assay may have obscured the modest rate enhancement observed in the dilution study. Finally, the rate of microtubule disassembly following a 1000-fold dilution of a steady-state reaction mixture into a 50% sucrose-buffer[3] was signficantly reduced by 0.167 M P_i compared to 0.111 M SO_4 (Figure 4). The results are similar to those reported previously (Carlier et al., 1988), except we found the effect of P_i was not instantaneous so there was appreciable disassembly before the rate was retarded.

In the studies described in Table I microtubules were both assembled and disassembled in the presence of P_i. We were also able to do a limited number of studies using a flow cell (Berg & Block, 1984) that allowed microtubules formed in the absence of added ligand to be studied in the presence of P_i. In two instances we were able to measure the rapid shortening rate of a microtubule formed in the absence of P_i, after the tubulin solution used for elongation was replaced with buffer containing P_i. In these cases the observed rates were comparable to that of microtubules that had both formed and disassembled in the presence of P_i. In another case a microtubule grown in the presence of P_i was sequentially exposed to a P_i-free and a P_i-containing tubulin solution, without a dramatic change in the rapid shortening rate when a catastrophe occurred. These observations are taken to indicate that the kinetic properties of microtubules formed in the presence of P_i are not dramatically different from those formed under ordinary reaction conditions.

P_i did not reduce the frequency of catastrophe (Figure 3; cf. k_c in reactions 3 and 4, Table I). Catastrophe was also frequent in the presence of P_i when the Mg^{2+} concentration was increased to 6 mM (Table I, cf. k_c in reactions 2 and 5).

[3] We also studied disassembly in the presence of sucrose and glycerol, because this corresponds to the conditions described in the earlier study of this reaction[2] (Carlier et al., 1988). In the presence of 50% sucrose and approximately 2 M glycerol there is no measurable disassembly in 1 h with or without 0.167 M P_i (data not presented).

Table III: Effect of P_i and SO_4 on Microtubule Assembly

added ligand	fraction of active tubulin[a] (SE)	critical concn[a] (SE) (μM)	fraction of inactive tubulin[b] (SE)
none	0.79 (0.01)	14.6 (0.6)	0.24 (0.03)
P_i	0.48[c] (0.02)	13.3 (0.8)	0.57[c] (0.06)
SO_4	0.58 (0.02)	14.4 (0.8)	0.20 (0.05)

[a] The amount of radioactive guanine nucleotide incorporated into polymer pelleted with an airfuge was determined in triplicate determinations, with 30, 40, and 65 μM tubulin. Results from a representative experiment are given here. The fraction of active tubulin and critical subunit concentration were determined from the slope and intercept on the ordinate, respectively, of a plot of moles of radioactive guanine nucleotide incorporated into polymer versus the total tubulin concentration. [b] Determined from the slope of a plot of protein concentration in the supernatant after centrifugation versus total tubulin used for assembly, under conditions where the total tubulin concentration is sufficient to allow net microtubule assembly. [c] We do not believe that the fractions of active and inactive tubulin are different in the presence of P_i and SO_4: In another typical experiment the fraction of active tubulin was 0.67 in a control, 0.53 in P_i, and 0.45 in SO_4, while the fraction of inactive tubulin was 0.31 in both P_i and SO_4.

P_i induced an increase in k_r at both microtubule ends (cf. k_r for reactions 3 and 1 in Table I). The increase with P_i does not result from a decrease in the free Mg^{2+} concentration, since with SO_4, which could be expected to induce a similar decrease in the free Mg^{2+} concentration,[4] there is no increase in k_r (cf. k_r in reactions 4 and 1 in Table I).

Both P_i and SO_4 decreased the second-order rate constant for microtubule elongation: it required 26 μM tubulin to produce rates equivalent to those seen with 16 μM tubulin in a control reaction (cf. v_e in reaction 1 and reactions 3 and 4, Table I). The reduction in v_e resulted from a decrease in the fraction of tubulin active for assembly. This decreased activity was indicated by a reduction by P_i of the slope of a plot of moles of radioactive guanine nucleotide incorporated into polymer versus total protein used for assembly (Table III). Also, in the tubulin subunit concentration range where microtubule assembly occurs, the slope of a plot of total protein used for assembly versus the amount of protein remaining in the supernatant after the steady-state reaction was centrifuged was increased by P_i (Table III); the slope of this plot is equal to the fraction of inactive protein. P_i's influence on assembly apparently results from an increased ionic strength, since comparable effects were produced by SO_4.[5] The effect of P_i on tubulin subunits is rapid, since the amount of radiolabeled guanine nucleotide incorporated into polymer was reduced within 10 min and remained approximately constant for 60 min (data not given).

Discussion

Possible Role of GDP–P_i Subunits in Microtubule Dynamic Instability. Microtubule dynamic instability has been ac-

[4] The Mg^{2+} concentrations will be significantly reduced by both P_i and SO_4. For example, with 6 mM total Mg^{2+} [the conditions used previously (Carlier et al., 1988)] addition of 0.15 M P_i is calculated to reduce the free Mg^{2+} to only 0.78 mM (Fabiato & Fabiato, 1979). This reduction is expected to be similar with P_i and SO_4, since the binding constant for Mg^{2+} is 158 M with SO_4 (Dunsmore & James, 1951) and 316 M with HPO_4^- (Greenwald et al., 1940).

[5] The decreased fraction of active tubulin is predicted [Figure 3 in Carlier (1989)] if tubulin–GDP does not assemble and exchange of GTP for GDP on tubulin–GDP–P_i (and tubulin–GDP–SO_4) subunits is slow. This possibility is, however, ruled out, since in the microscopy studies, where there is no significant accumulation of tubulin–GDP subunits (with or without bound P_i or SO_4), the elongation rate is reduced. This means that the effect of P_i and SO_4 is on the elongation reaction, rather than on nucleotide exchange.

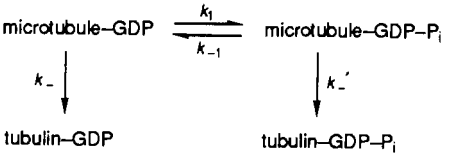
counted for by assuming that microtubules are structurally and kinetically heterogeneous. According to one model, the heterogeneity consists of a relatively short run of tubulin–GTP subunits at each end of a core of tubulin–GDP subunits that constitutes the bulk of the microtubule (Carlier & Pantaloni, 1981; Kirschner & Mitchison, 1986). The GTP in terminal subunits is not yet hydrolyzed since these subunits have only recently been incorporated into polymer. In an alternate mechanism, based on studies of effects of P_i and P_i analogues (Carlier et al., 1988), the heterogeneity is derived from a cap of tubulin–GDP–P_i subunits at ends of the tubulin–GDP core. Although P_i generated by hydrolysis of subunit-associated GTP is presumed to be reversibly dissociable from tubulin–GDP subunits in the microtubule, tubulin–GDP–P_i subunits are primarily located at ends because P_i binding is relatively weak (K_d = 0.025 M; Carlier et al., 1988), and the concentration of P_i generated by GTP hydrolysis in most studies of microtubule dynamics is ordinarily only about 100–200 μM. As a result, under ordinary assay conditions P_i dissociation from microtubule subunits is essentially irreversible. However, if P_i dissociation is slow, recently polymerized subunits will have not yet lost P_i formed by GTP hydrolysis, so that there will be a cap of tubulin–GDP–P_i subunits at the ends of a much larger core of tubulin–GDP subunits. Thus, the two models differ as to whether the cap consists of subunits that have not yet cleaved GTP or not yet dissociated the P_i generated by hydrolysis. The models are similar in assuming that terminal subunits dissociate from ends more slowly than tubulin–GDP subunits. It was recently found (Caplow et al., 1988) in studies of the rate of microtubule disassembly following a small temperature drop that terminally located subunits dissociate more slowly than internal subunits; the chemical basis of this behavior was not established.

The tubulin–GTP and tubulin–GDP–P_i cap models make distinct and different predictions that allow them to be differentiated. Because the GDP in tubulin–GDP subunits in the microtubule is not exchangeable, while P_i in tubulin–GDP–P_i subunits is exchangeable (Carlier et al., 1988), the heterogeneity in the microtubule's structure and reactivity would not be eliminated by incubation with a high concentration of GTP, but would be eliminated by a high concentration of added P_i. In the presence of a high P_i concentration, interior tubulin–GDP subunits will be converted to tubulin–GDP–P_i subunits, so that terminal and internal subunits are equivalent. This can be expected to eliminate the heterogeneity in reaction rates that produces dynamic instability behavior. As a result, catastrophes are expected to be abolished in the presence of a high concentration of P_i. Also, because tubulin–GDP–P_i subunits have been reported to dissociate from microtubule ends more slowly than tubulin–GDP subunits (Carlier et al., 1988), it is expected the rate of shortening of microtubules that are in the disassembly regimen will be reduced by P_i.

Observed Effect of P_i on Microtubule Dynamics. We found no difference in the microtubule catastrophe frequency (k_c) in the presence of either P_i or SO_4 (reactions 3, 4, Table I). It is expected that catastrophes would not occur if the microtubule consisted predominantly of tubulin–GDP–P_i subunits, so that terminal tubulin–GTP and internal subunits had similar slow dissociation rates. We also found that P_i did not reduce the subunit dissociation rate during rapid shortening (v_{rs}). This reaction is expected to be slower if P_i binds to and stabilizes subunits in the microtubule core.[6]

In evaluating these effects it is necessary to consider the possibility that P_i binding to tubulin–GDP subunits in microtubules is sufficiently slow, under the conditions used in our microscopy studies, that the microtubules did not equilibrate with the P_i added to reaction mixtures. Microtubules had a relatively short mean lifetime before undergoing catastrophic disassembly (185–526 s for reactions 3 and 4 in Table I), and this may be too short for equilibration with P_i. We suggest this possibility can be ruled out since, in control reactions without added P_i, the shortening rate of the portion of the microtubule formed a minute or two before a catastrophe was not noticeably different from that of lengths more distal from the end. Assuming that P_i acts to decrease the rate of dissociation of subunits, the uniform shortening rate indicates that P_i dissociation from tubulin–GDP–P_i subunits derived by GTP hydrolysis is largely complete within about 1 min. Since the rate of P_i dissociation and association are equal when the P_i concentration is equal to K_d, the pseudo-first-order binding of P_i is expected to be at equilibrium within about 1 min, when the concentration is 0.025 M. At the higher P_i concentrations used here (0.167 M) P_i binding will be at equilibrium in less than 1 min, which is less than the mean lifetime of the microtubules. Thus, if 0.167 M P_i could influence microtubule dynamics, we would expect to see this effect, despite the relatively short mean lifetime of the microtubules under our reaction conditions. Additional evidence

[6] In the previous study of the effect of P_i on microtubule dynamics the rate of subunit dissociation from microtubules in the rapid shortening regimen is relatively slow (Carlier et al., 1988), while under the conditions used here, where dynamic instability is manifested, this rate is exceedingly rapid. This raises the question whether P_i can be expected to be as effective an inhibitor when disassembly is rapid. To deal with this we have analyzed a scheme for microtubule rapid shortening that takes into account the rates of both subunit and ligand dissociation. Binding of P_i to tubulin–GDP subunits in microtubules under conditions of rapid shortening is described in



It is assumed that P_i binding occurs to the nucleotide binding site in all subunits, since the binding reaction is simply the reverse of the P_i dissociation reaction and the principle of microscopic reversibility (detailed balance) requires that the association reaction can occur. The rate of disassembly for the reaction outlined in the above scheme is given by

$$v_{rs} = \frac{k_1k_-' + k_-k_{-1} + k_-k_-'}{k_1 + k_{-1} + [(k_1k_- + k_{-1}k_-')/(k_1 + k_{-1})]} \qquad \text{(MT)}$$

Binding of P_i to interior as well as terminal tubulin–GDP subunits provides a second way to have a tubulin–GDP–P_i subunit at a shortening end: by discovery of an interior tubulin–GDP–P_i subunit, after dissociation of overlying tubulin–GDP subunits. The discovery path will have an major impact on the microtubule shortening rate if the subunit dissociation reaction is rapid relative to the P_i association/dissociation reactions. This point is illustrated by comparing the cases where the rate of subunit dissociation is slow (k_- = 1, k_-' = 0.1) or rapid (k_- = 1000, k_-' = 100) relative to the rate of P_i dissociation (10 s^{-1}). If subunit dissociation is slow relative to P_i dissociation, the rate is reduced about 2-fold when the P_i concentration is equal to K_d (0.025 M, for the example considered). However, if subunit dissociation is rapid relative to P_i dissociation, then P_i is a more effective inhibitor, since tubulin–GDP–P_i subunits that are discovered at the microtubule end by dissociation of P_i-free subunits act to inhibit the rate. As a result, P_i inhibition is much greater; the rate is reduced 2-fold with 0.0035 M P_i in the hypothetical reaction described here. In summary, if P_i binds to tubulin–GDP subunits and inhibits microtubule disassembly ($k_- > k_-'$), this effect should be readily detectable with P_i concentrations $\geq K_d$. Furthermore, we have maximized the likelihood of observing an effect by working under conditions where the subunit dissociation rate is high.

that P_i binding is relatively rapid is provided by the fact that in the earlier study of the effect of P_i on the the rate of dilution-induced disassembly (Carlier et al., 1988) the microtubules did not require incubation with P_i prior to dilution to see an effect on the disassembly rate. This observation could not, however, be confirmed here: we found (Figure 4) that P_i stabilization of microtubules in sucrose buffer requires several seconds. The most convincing evidence that our failure to observe an effect of P_i on microtubule stability did not result from slow binding to microtubules was derived from a macroscopic assay of microtubule dynamics (Table III), in which its effect on the critical subunit concentration was determined. In this experiment microtubules were incubated with P_i (or SO_4) for 10–60 min. Thus, even if some microtubules are short-lived because of dynamic instability behavior, it is expected that if P_i were reactive with tubulin–GDP subunits in microtubule, a progressive trapping and stabilization of microtubules would occur during this long incubation. Consequently, our observation that P_i did not reduce the steady-state subunit concentration indicates that P_i does not bind to and stabilize microtubules.

We next make a quantitative estimate of the stabilization that is expected if P_i were able to react with and stabilize microtubules. For a dynamic instability model, the steady-state subunit concentration is equal to that necessary to make the rate of net elongation (v_e) times average time spent in the elongation phase ($1/k_c$) equal to the product of the rate of subunit loss during rapid shortening (v_{rs}) times average time spent in the rapid shortening phase ($1/k_r$); i.e., $v_e(\text{tubulin})_{\text{steady state}}/k_c = v_{rs}/k_r$. Assuming that tubulin–GDP–P_i subunits dissociate at the rate of tubulin–GTP subunits and using the previously determined rate constants (Walker et al., 1988), it is calculated that the steady-state concentration would increase 2.3-fold if P_i were to bind to the tubulin–GDP subunits in the microtubule. That is (at 37 °C), the calculated steady-state concentration was 10.9 μM [Figure 9 in Walker et al. (1988)], and this would decrease to 5.1 μM if dynamic instability were to be eliminated by P_i. We found, instead, that after correcting for the effect of P_i on the fraction of our beef tubulin that is active, the steady-state subunit concentration was not different from that of the control reaction (Table III).

Summary and Conclusion. Using buffer conditions that support microtubule dynamic instability and the maximum practical P_i concentration, we have failed to observe any of the effects predicted if P_i were to bind to tubulin–GDP subunits in the microtubule and reduce the rate of subunit dissociation. Except for the reaction in 50% sucrose buffer, we found no evidence of interaction of P_i with subunits in microtubules. It is concluded that either P_i has very low affinity for tubulin–GDP subunits in microtubules under our reaction conditions or binding occurs and this does not alter the rate constant for subunit dissociation.

With regard to the former possibility, while such an effect may account for our failure to observe an influence of P_i on k_c or k_-, it should be noted that this hypothetical reduction in the binding affinity of P_i to tubulin–GDP subunits in the microtubule would be likely to come about because P_i dissociates from these subunits more rapidly. The effect of such rapid dissociation of P_i would act to nullify the postulated effects of P_i on microtubule dynamics, since the tubulin–GDP–P_i cap model is predicted upon P_i dissociation being slow. Thus, if the binding of P_i to microtubules is weak under conditions where dynamic instability is observed, it is unlikely that P_i association with the microtubule can generate dynamic instability behavior. Our results do not support the hypothesis that tubulin–GDP–P_i subunits contribute to dynamic instability behavior.

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