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Effects of Organic Acids on Tubulin Polymerization and Associated Guanosine 5'-Triphosphate Hydrolysis†

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ABSTRACT: We have examined the effects of a number of organic anions, which stabilize tubulin, on tubulin polymerization, associated GTP hydrolysis, and polymer morphology. While microtubule-associated proteins, as well as glycerol, induced formation of typical microtubules in a reaction coupled to GTP hydrolysis at an initial 1:1 stoichiometry, the organic anions had varying effects. Only 2-(*N*-morpholino)ethanesulfonate induced formation of structures with the morphology of microtubules. With glutamate, fructose 1,6-bisphosphate, piperazine-*N*-*N*'-bis(2-ethanesulfonate), glutarate, and glucose 1-phosphate, the predominant structures formed were sheets of parallel protofilaments rather than microtubules. Creatine phosphate induced the formation of clusters of rings. GTP hydrolysis was closely coupled to polymerization only with glutamate. With creatine phosphate, there was minimal GTP hydrolysis. With all other organic anions, GTP hydrolysis substantially exceeded polymerization at all time points, with the onset of hydrolysis significantly preceding the onset of turbidity development. Nevertheless, the rate of GTP hydrolysis was a sigmoidal function of tubulin concentration under all conditions examined, suggesting that tubulin–tubulin interactions are required for hydrolysis. All anion-induced reactions were temperature dependent and cold reversible, but only the creatine phosphate induced reaction was not inhibited by GDP, Ca^{2+}, or colchicine and did not require GTP.

We have observed that a number of organic anions exert impressive stabilizing effects on the colchicine binding activity of tubulin (Hamel & Lin, 1980, 1981b), and most of them also induce purified tubulin to polymerize in a temperature-dependent, cold-reversible reaction (Hamel & Lin, 1980, 1981a). We have characterized these properties most extensively with glutamate and have exploited them to develop a high-yield, large-scale purification of tubulin free of nucleosidediphosphate kinase and ATPase activities (Hamel & Lin, 1981a,b). In an effort to determine whether there was a common basis for the stabilizing and polymerizing properties of the various effective anions (carboxylates, phosphates, and sulfonates), we have now examined their effects on the morphology of the polymerized tubulin and on GTP hydrolysis associated with polymerization.

Materials and Methods

Materials

Monosodium glutamate was obtained from Grand Island, GTP, 2-(*N*-morpholino)ethanesulfonate (Mes),[1] and CP were from Sigma, Pipes was from Calibiochem, and [α-^{32}P]GTP was from Amersham. Organic anions were obtained as the sodium salts or free acids, and all stock solutions were adjusted to pH 6.6 with NaOH or HCl. GTP was repurified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25.

Proteins. Purified calf brain tubulin and heat-treated MAP's were prepared as described previously (Hamel & Lin, 1981a; Hamel et al., 1981). The tubulin used here was free of nucleosidediphosphate kinase and ATPase activities, electrophoretically homogeneous, freed of unbound nucleotide by gel filtration chromatography (Hamel & Lin, 1981a), and concentrated as described previously (Hamel et al., 1981a). It was stored at 30.5 mg/mL in 1.0 M glutamate in liquid nitrogen. Analysis of the bound nucleotide has demonstrated that the tubulin contains 1.9 mol of guanine nucleotide per mol of protein, half of which is GTP and half GDP. We have assumed that the GTP is bound in the nonexchangeable site and the GDP in the exchangeable site since a polymerization step followed by homogenization of the polymer in a GTP-free solution was a late step in the purification, and since further polymerization steps require at least stoichiometric amounts of GTP (Hamel & Lin, 1981ac).

The heat-treated MAP's (Fellous et al., 1977) had little ATPase or nucleosidediphosphate kinase activity (Hamel et al., 1981) as well as little GTPase activity (David-Pfeuty et al., 1979).

Methods

Tubulin polymerization was followed turbidimetrically (Gaskin et al., 1974) as described previously (Hamel & Lin,

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[1] Abbreviations: MAP's, microtubule-associated proteins; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonate); Mes, 2-(*N*-morpholino)ethanesulfonate; CP, creatine phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetate.

1981a). Thin-layer chromatography on poly(ethyleneimine)–cellulose and autoradiography were used to assay GTPase activity by measuring the formation of [α-^{32}P]GDP from [α-^{32}]GTP (Hamel & Lin, 1981a). When GTP hydrolysis and turbidity were followed simultaneously by removing aliquots of reaction mixtures from cuvettes, the extent of polymerization in a reaction was determined by centrifugation as described previously (Hamel et al., 1981). The percent tubulin polymerized was then converted to nanomoles per milliliter of reaction, based on a molecular weight of 110 000, and the turbidity plateau was placed at this level on the "GDP formed" scale. In the experiments presented here, the degree of reversibility was assessed by chilling the reactions to 0 °C, with the final turbidity readings shown in the individual experiments. With all organic anions, experiments were performed at protein concentrations in which the final turbidity reading was a linear function of protein concentration. The data in all GTP hydrolysis experiments are expressed as nanomoles of GDP formed per milliliter of reaction.

Electron microscopy was performed as described previously (Hamel et al., 1981), except that the pellet of polymerized material formed in 1.0 M glutamate was fixed in a solution containing 10% formalin, 1.0 M glutamate, and 0.1 mM GTP and postfixed in 1% osmium tetraoxide in the same solution without formaldehyde. Glutaraldehyde was unsuitable for use in fixing material polymerized in high concentrations of glutamate because a rapid chemical reaction between glutaraldehyde and glutamate completely disrupted the morphology of the polymer.

Results

Figures 1–3 present studies in which tubulin polymerization and GTP hydrolysis were examined simultaneously in a variety of systems, with comparisons to the electron microscopic appearance of the polymerized tubulin. In all cases, the polymerization reactions were temperature dependent and cold reversible.

Of all the organic anions we have examined, microtubules predominate only in Mes (Figure 1B,C) Double and multiple tubules, many resembling ciliary outer doublet tubules, as well as some open forms, were seen more frequently with 0.3 mM GTP (Figure 1B) than with 1 mM nucleotide (Figure 1C). This may be because polymerization occurs more slowly at the lower nucleotide concentration (data not presented). The time course study of Figure 1A, however, demonstrates that there is significant uncoupling of GTP hydrolysis from polymerization in Mes, hydrolysis beginning after a much shorter lag period. The GTPase reaction occurred at a linear rate of about 0.7 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$ both before polymerization began and throughout its course.

The Mes-dependent polymerization reaction has a relatively high critical concentration of about 0.8 mg/mL (with the other organic anions described here, critical concentrations are 0.1–0.2 mg/mL; data not presented) and requires a higher GTP concentration. We had previously (Hamel et al., 1981) confirmed the results of David-Pfeuty et al. (1977) that MAP-dependent tubulin polymerization was initially closely coupled to GTP hydrolysis with a tubulin concentration of 1 mg/mL. To exclude the possibility that the results with Mes were simply caused by the higher tubulin and GTP concentrations required, we repeated the MAP-dependent experiment at the same tubulin and GTP levels (Figure 1D). Results comparable to those previously reported (David-Pfeuty et al., 1977; Hamel et al., 1981) were obtained: a burst of GTP hydrolysis [initial rate about 0.7 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$] occurred simultaneously with the onset of polymerization at a 1:1 stoichiometry, followed by a slower, linear rate of hydrolysis [about 0.2 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$] as polymerization approached its plateau. Figure 1E confirms that microtubules were formed in this MAP-dependent reaction.

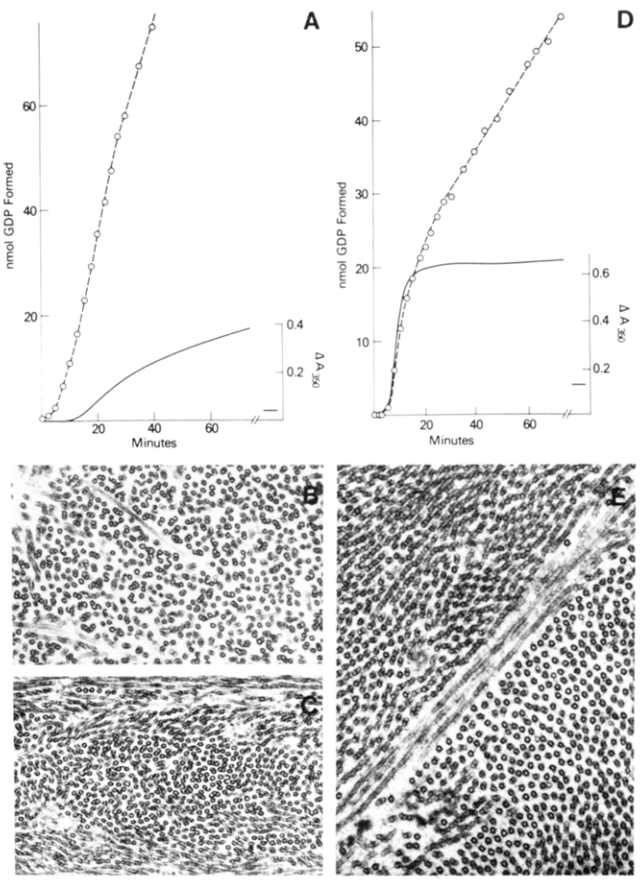

FIGURE 1: Tubulin polymerization dependent on Mes or heat-treated MAP's. Each reaction mixture contained 3 mg/mL tubulin, 0.3 mM GTP or [α-^{32}P]GTP, and either 1.7 M Mes or 1 mg/mL heat-treated MAP's and 0.1 M glutamate. In the time course studies of panels A and D, reaction volume was 1.3 mL. The solid line represents turbidity; the open circles represent GTP hydrolysis. The short line to the right of the break in the axis represents the turbidity reading following cold depolymerization. At the indicated times, 5-μL aliquots were removed from the cuvettes and analyzed for GTP hydrolysis as described elsewhere (Hamel & Lin, 1981a). At the end of the timed study, 0.5 mL of each sample was centrifuged at 50 000 rpm for 30 min in a Beckman Ti 50 rotor to determine the amount of protein polymerized, and the turbidity plateau was placed at this value on the GDP formed scale. In Mes, 63% of the protein was polymerized, or 17.2 nmol/mL tubulin, and with MAP's, 77% was polymerized, or 21 nmol/mL tubulin. (Polyacrylamide gels of the total reaction mixture and of the postcentrifugation supernatant in the MAP's experiment demonstrated that the percent tubulin was almost identical in the two preparations, indicating tubulin and MAP's were polymerized in a ratio approximately equal to their ratio in the original reaction mixture.) (A) Time course of turbidity development and GTP hydrolysis in 1.7 M Mes. (B) Thin section of tubulin polymerized in Mes (29 160×). (C) Thin section of tubulin polymerized in Mes with 1 mM instead of 0.3 mM GTP (29 160×). (D) Time course of turbidity development and GTP hydrolysis with MAP's. (E) Thin section of tubulin polymerized with MAP's (29 160×).

In contrast to the tubules induced by Mes, the polymer formed in most other organic acids consists of a variety of aberrant forms, predominantly sheets of ribbons of parallel protofilaments which varied substantially in length and width. Panels B and C and panels E and F of Figure 2 present electron micrographs of the structures induced by glutamate and Pipes, respectively, while Figure 2A,D presents time course studies of polymerization and GTP hydrolysis. Closed

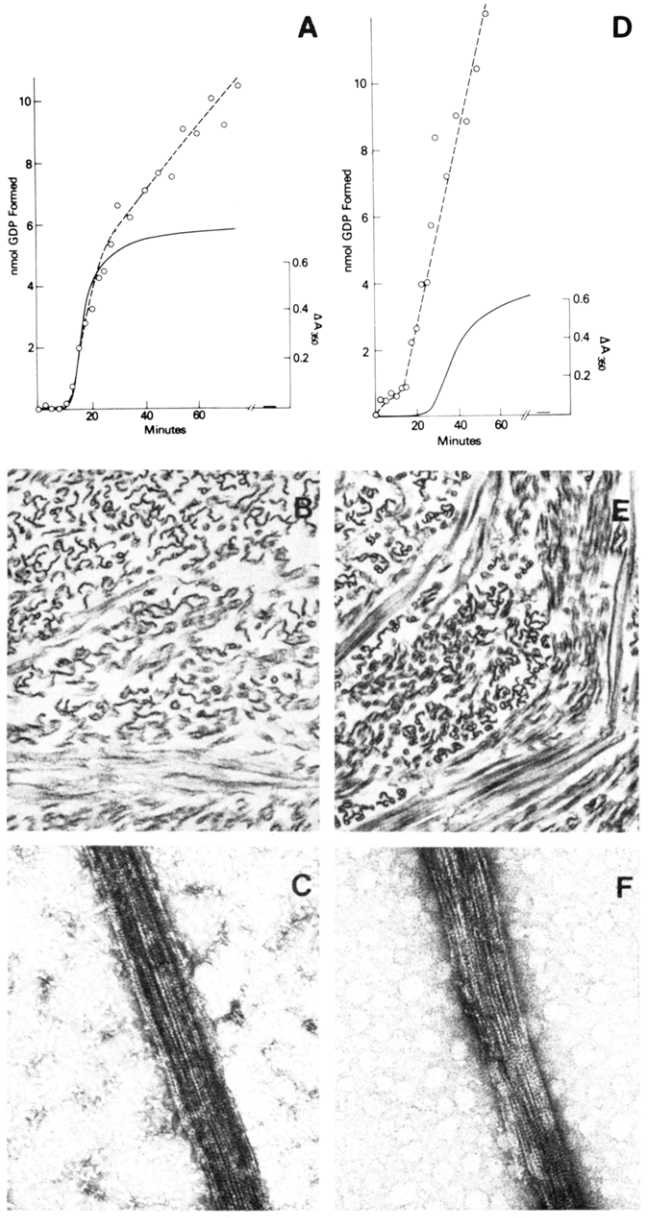

FIGURE 2: Tubulin polymerization dependent on glutamate or Pipes. Each reaction mixture contained 0.8 mg/mL tubulin, 0.1 mM GTP or [α-^{32}P]GTP, and either 1.0 M glutamate or 0.8 M Pipes. Further conditions and methodology were as described in Figure 1. (A) Time course of turbidity development and GTP hydrolysis in 1.0 M glutamate; 81% of the tubulin was polymerized, or 5.9 nmol/mL. (B) Thin section of tubulin polymerized in glutamate (35 100×). (C) Negatively stained preparation of tubulin polymerized in glutamate (101 400×). (D) Time course of turbidity development and GTP hydrolysis in 0.8 M Pipes; 50% of the tubulin was polymerized, or 3.6 nmol/mL. (E) Thin section of tubulin polymerized in Pipes (35 100×). (F) Negatively stained preparation of tubulin polymerized in Pipes (101 400×).

structures similar to microtubules or double tubules were seen more frequently in Pipes than in glutamate.

Hydrolysis was closely coupled to polymerization, however, only in glutamate. As described previously (Hamel & Lin, 1981a), the relationship of the glutamate-dependent hydrolytic and polymerization reactions closely resembles that observed with MAP's. On the other hand, the reactions dependent on Pipes resemble the Mes-dependent reactions: the lag period prior to the onset of hydrolysis was significantly shorter than the lag prior to polymerization. With Pipes, hydrolysis occurred only with a sustained linear rate of about 0.4 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$, while in the glutamate-dependent reaction the more rapid initial rate of 0.5 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$ was followed by a slower rate of 0.1 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$ as polymerization approached its plateau.

We have also found that the preparations of fructose 1,6-bisphosphate, glucose 1-phosphate, and glutarate which stabilize the colchicine-binding activity of tubulin (Hamel & Lin, 1981b) also induce formation of polymer with predominantly sheet morphology. In these cases, the relationship of polymerization to GTP hydrolysis differed little from that observed in Pipes, with hydrolysis beginning prior to turbidity development (data not presented). The interpretation of the studies with the two sugar phosphates, however, is complicated by their contamination with high levels of divalent cations (Hamel & Lin, 1981b) which themselves have been shown to have significant effects in inducing tubulin polymerization (Larsson et al., 1976; Herzog & Weber, 1977; Gaskin & Kress, 1977).

A third pattern of tubulin polymerization, nucleotide hydrolysis, and polymer morphology was observed in CP (Figure 3). Cold-reversible turbidity development occurred after a typical lag period, but, unlike the previously described systems, there was minimal associated GTP hydrolysis (Figure 3). The polymer formed had a strikingly different morphology as well (Figure 3B,C). Thin sections of polymerized material had the appearance of an extensive array of rings, as of a slice taken through a meshwork (Figure 3B). Negatively stained material, however, suggested small clusters of adjacent rings rather than more extensive networks (Figure 3C). In addition, scattered sheets of protofilaments were observed (data not presented). At 0 °C, only a few single rings were seen (Figure 3D).

The CP-induced polymerization reaction was unique in other ways as well. The reactions dependent on Mes, Pipes, fructose 1,6-bisphosphate, glutarate, and glucose 1-phosphate required GTP and were inhibited by colchicine, GDP, or Ca^{2+} (data not presented), like the reaction with glutamate (Hamel & Lin, 1981a). We could demonstrate none of these effects with CP. Most notably, the reaction was entirely independent of GTP and unaffected by GDP (data not presented).

As this study was nearing completion, Carlier & Pantaloni (1981) published distinctly different results with glycerol-induced tubulin polymerization. These workers reported that a substantial lag occurred between initial turbidity development and later GTP hydrolysis when the two reactions were compared on a molar basis. Since we had found contrary results in preliminary experiments with glycerol under somewhat different conditions, we reexamined turbidity development and GTP hydrolysis simultaneously under conditions similar to those described by Carlier & Pantaloni (1981).

Figure 4 presents our results. For elimination of the effects of glutamate, our tubulin preparation, which is free of unbound nucleotide and contains GDP in the exchangeable site (Hamel & Lin, 1981a), was dialyzed against the glycerol solution (without GTP) described by Carlier & Pantaloni (1981). If radioactive GTP was added just prior to beginning the incubations (Figure 4A, curve 1), the onsets of polymerization and hydrolysis were simultaneous at an initial 1:1 stoichiometry. The initial burst of hydrolysis [0.5 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$] was followed by a somewhat slower, linear reaction [0.4 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$].

Since Carlier & Pantaloni (1981) had included GTP throughout their purification of tubulin, with a final dialysis against a GTP-containing solution, their tubulin undoubtedly contained GTP in the exchangeable site, as well as free GTP, prior to their addition of [γ-^{32}P]GTP. If the radioactive nucleotide was not freely exchangeable as assumed, this could

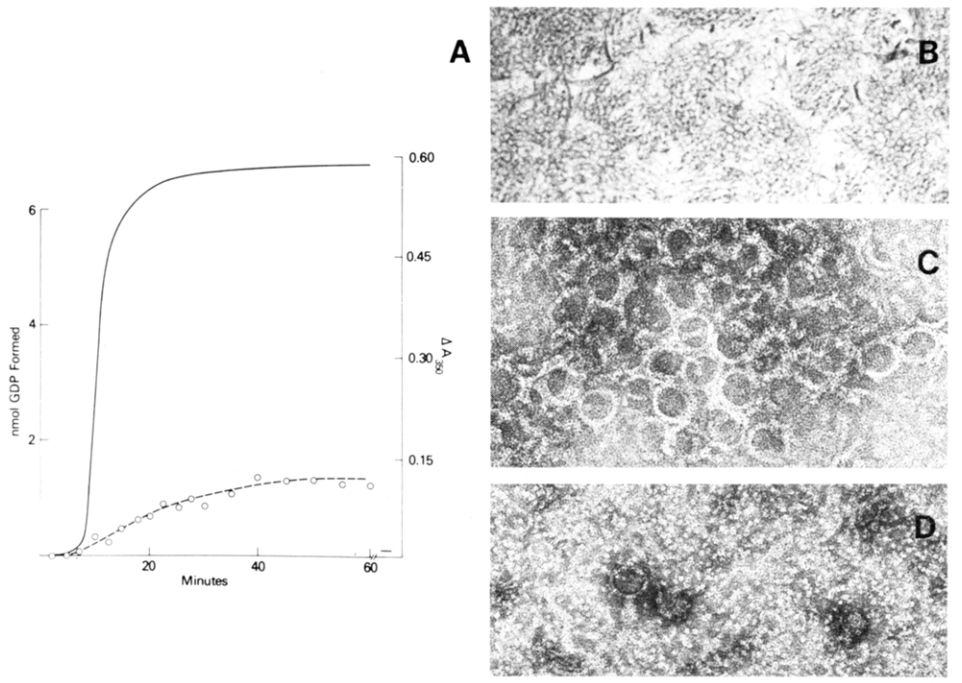

FIGURE 3: Tubulin polymerization dependent on CP. Each reaction mixture contained 0.8 mg/mL tubulin, 0.1 mM GTP or [α-^{32}P]GTP, and 0.9 M CP. Further conditions and methodology were as described in Figure 1. (A) Time course of turbidity development and GTP hydrolysis; 93% of the tubulin was polymerized, or 6.8 nmol/mL. (B) Thin section of polymerized tubulin (37 800×). (C) Negatively stained preparation of polymerized tubulin (109 200×). (D) Negatively stained preparation of tubulin incubated at 0 °C for 1 h (109 200×).

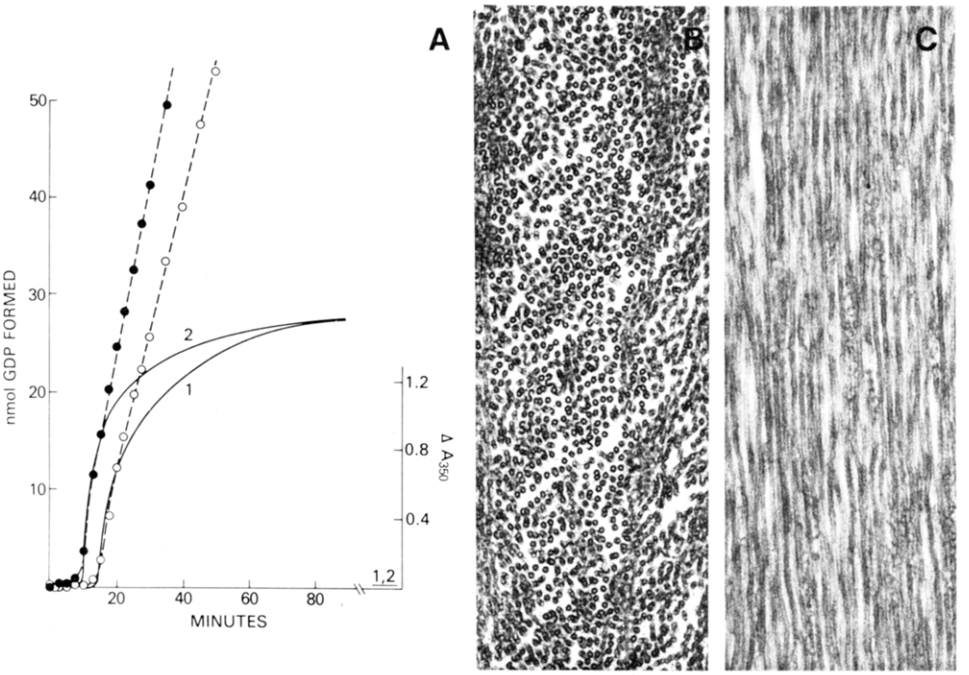

FIGURE 4: Tubulin polymerization dependent on glycerol and Mg^{2+}. Each reaction mixture contained 4.0 mg/mL tubulin, 3.4 M glycerol, 6 mM $MgCl_2$, 0.05 M Mes (pH 6.6), 0.5 mM EGTA, and GTP as indicated. Prior to use in this experiment, the tubulin was dialyzed for 4 h at 0 °C against a solution containing 3.4 M glycerol, 6 mM $MgCl_2$, 0.05 M Mes (pH 6.6), and 0.5 mM EGTA. Further conditions and methodology were as described in Figure 1. (A) Time course of turbidity development and GTP hydrolysis. In the experiment described by curve 1 (the solid line representing turbidity, the open circles representing GTP hydrolysis), 200 μM [α-^{32}P]GTP was added immediately before beginning the incubation; 78% of the tubulin was polymerized, or 28.4 nmol/mL. In the experiment described by curve 2 (the solid line representing turbidity, the closed circles representing GTP hydrolysis), 36 μM nonradioactive GTP (equimolar with tubulin) was added 3 h prior to the incubation at 37 °C, and then 200 μM [α-^{32}P]GTP was added immediately before beginning the incubation; 77% of the tubulin was polymerized, or 28.0 nmol/mL. (B and C) Thin sections of tubulin polymerized under conditions identical with those described in Figure 4A for curve 1 (37 800×).

explain the difference in data. To test this possibility, we preincubated tubulin with equimolar nonradioactive GTP at 0 °C prior to the addition of [α-^{32}P]GTP. We found (Figure 4A, curve 2), however, that the two reactions still occurred simultaneously with an initial 1:1 stoichiometry, although their onset was somewhat earlier.

The reason for the discrepancy between our results and those of Carlier & Pantaloni (1981) is unclear, but it should be noted that the basic experimental observation is the same: the maximum rate of GTP hydrolysis occurs during the early

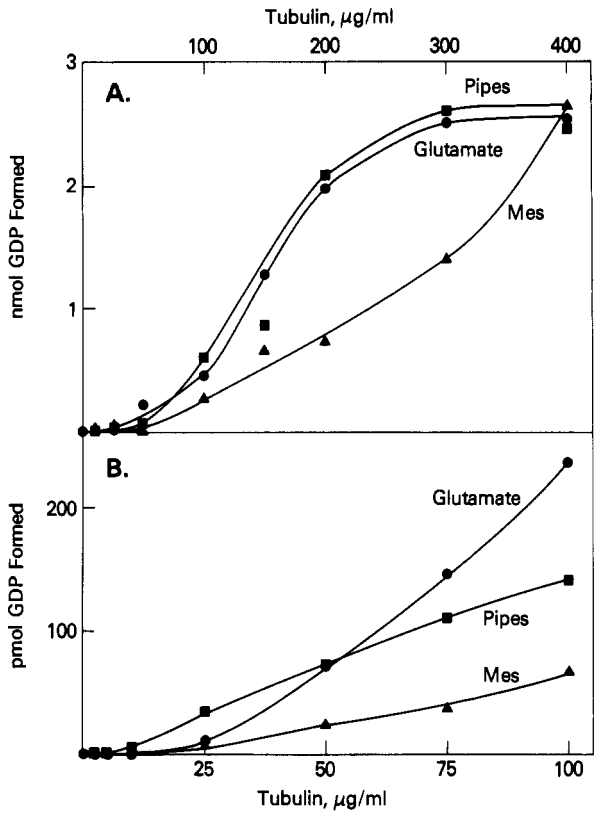

FIGURE 5: Effect of tubulin concentration on GTP hydrolysis. Each 50-μL reaction mixture contained the indicated concentrations of tubulin and [α-^{32}P]GTP, as well as one of the following as indicated: 1.0 M glutamate (●); 0.8 M Pipes (■); or 1.7 M Mes (▲). At the indicated times, a 5-μL aliquot of each reaction mixture was analyzed for GTP hydrolysis as described elsewhere (Hamel & Lin, 1981a). (A) Higher tubulin concentrations. Each reaction mixture contained 20 μM [α-^{32}P]GTP and was incubated for 30 min at 37 °C. (B) Lower tubulin concentrations. Each reaction mixture contained 1 μM [α-^{32}P]GTP and was incubated for 60 min at 37 °C.

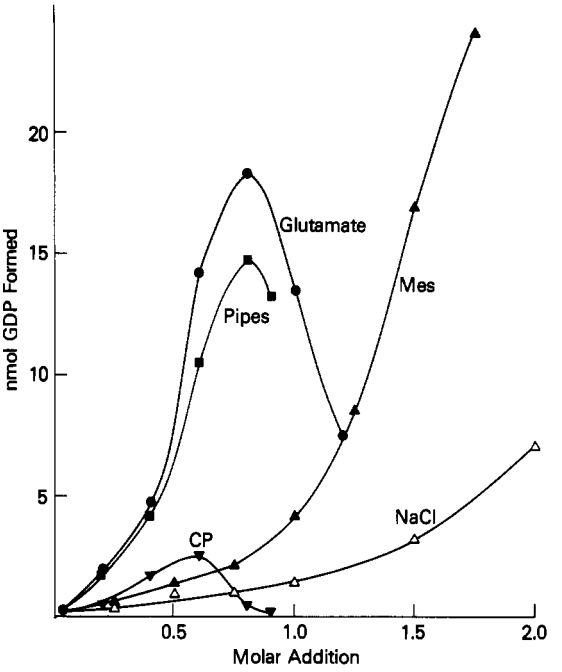

FIGURE 6: Effect of anion concentration on GTP hydrolysis. Each 30-μL reaction mixture contained 1 mg/mL tubulin, 0.1 mM [α-^{32}P]GTP, and the indicated concentration of glutamate (●), Pipes (■), Mes (▲), CP (▼), or NaCl (△). After 30 min at 37 °C, a 5-μL aliquot of each reaction mixture was analyzed for GTP hydrolysis as described elsewhere (Hamel & Lin, 1981a).

phases of turbidity development. Since our tubulin preparation contains no unbound nucleotide initially and the complete reaction mixture was analyzed, our conversion of crude data to nanomoles of GDP formed was simply based on the percent of [α-^{32}P]GTP converted to [α-^{32}P]GDP. The methodology of Carlier & Pantaloni (1981) was significantly more complex. Moreover, despite the elegance of their kinetic analysis, Carlier & Pantaloni (1981) did not demonstrate the presence of [γ-^{32}P]GTP on polymerized tubulin, a critical requirement for the model they presented. It seems unlikely that the tubulin itself has different properties: as shown in Figure 4B,C our preparation formed typical microtubules in glycerol and Mg^{2+} (Lee & Timasheff, 1975).

The apparent uncoupling of the onset of polymerization from the onset of GTP hydrolysis under many of the conditions described here raised a significant question: did GTP hydrolysis require tubulin–tubulin interactions which were spectroscopically inapparent? We had also previously observed that reduced GTP hydrolysis occurred at lower glutamate concentrations and at substoichiometric GTP concentrations at which no polymerization occurred (Hamel & Lin, 1981a). As a preliminary approach to this problem, we examined GTP hydrolysis as a function of tubulin concentration under a variety of conditions, some of which are presented in Figure 5. If single tubulin molecules hydrolyzed GTP, a graph of the reaction rate should be linear and pass through the origin as a function of tubulin concentration. A sigmoidal pattern should be obtained, however, if hydrolysis requires a tubulin–tubulin interaction. In every case, including those presented in Figure 5, sigmoidal patterns were observed. Little or no GTP hydrolysis occurred at the lower tubulin concentrations. This is in distinct contrast to the stimulation of GTP hydrolysis by colchicine, which David-Pfeuty et al. (1979) reported did not require tubulin–tubulin interactions.

Figure 6 demonstrates the variation among organic anions when GTP hydrolysis was examined as a function of anion concentration. The relatively sluggish hydrolysis obtained with NaCl demonstrates that ionic strength itself was a minor factor in stimulating tubulin's GTPase activity.

Finally, we were surprised by the apparent lack of a requirement for divalent cations in the polymerization reactions induced by organic acids, as most workers have reported a Mg^{2+} requirement for polymerization in other systems [for a review, see Timasheff & Grisham (1980)]. We therefore analyzed the preparations used here by atomic absorption spectroscopy for Mg^{2+} as well as Zn^{2+} (Larsson et al., 1976; Gaskin & Kress, 1977) and Mn^{2+} (Buttlaire et al., 1980) contamination. These analyses indicated that a 0.8 M solution of Pipes contained 0.01 mM Mg^{2+}, but no Zn^{2+} or Mn^{2+}; a 1.7 M solution of Mes, 0.07 mM Mg^{2+}, but no Zn^{2+} or Mn^{2+}; a 0.9 M solution of CP, 0.12 mM Mg^{2+}, but no Zn^{2+} or Mn^{2+}; and a 1.0 M solution of glutamate, 0.77 mM Mg^{2+}, 0.01 mM Zn^{2+}, and 0.05 mM Mn^{2+}; the repurified GTP did not contain significant amounts of any of these cations.

These findings leave open the question of whether Mg^{2+} or another cation is absolutely required in these systems, but the Mg^{2+} contaminantion in both the Pipes- and Mes-dependent systems was little more than stoichiometric with tubulin. Additional Mg^{2+} had no effect on Mes-induced polymerization and slightly stimulated the Pipes-induced reaction (data not presented). We have also found that glutamate-dependent polymerization is not greatly affected by EDTA and that the threshold concentration of glutamate required for tubulin polymerization is not altered by additional Mg^{2+} (Hamel & Lin, 1981a). These findings exclude the possibility that the effect of glutamate results simply from the presence of a

minimal amount of Mg^{2+} in the reaction mixture.

Discussion

We have thus far observed three distinct morphological forms of tubulin polymer induced by organic anions. Only Mes caused tubulin to polymerize into microtubules, and the Mes-dependent reaction was also unique in the high critical concentration of tubulin required. Most other solvent systems in which purified tubulin forms microtubules also have relatively high critical concentrations of the protein (Lee & Timasheff, 1975; Himes et al., 1977; Herzog & Weber, 1977, 1978).

Most of the anion preparations we have studied (glutamate, Pipes, fructose 1,6-bisphosphate, glutarate, and glucose 1-phosphate) induce the formation of polymer in which sheets of parallel protofilaments are prominent, with closed tubular structures being seen with varying frequency. It is perhaps of importance that with the exception of glucose 1-phosphate these were all bifunctional anions. Similar structures have been reported by other workers in response to a number of solvent conditions [cf. Larsson et al. (1976), Gaskin & Kress (1977), Herzog & Weber (1978), and Himes et al. (1979)] which have usually included sulfonate buffers.

Suzaki et al. (1978) have reported marked increases in viscosity when microtubule protein was polymerized in the presence of high concentrations of glutamate. These workers interpreted their results to indicate changes in the flexibility of microtubules. Our results, however, clearly demonstrate that the increased turbidity, as well as viscosity, observed with glutamate is caused by formation of sheets of protofilaments rather than subtle changes in microtubule structure. Furthermore, it is clear that the effect of glutamate is attributable to its interactions with tubulin, regardless of whether or not MAP's are present.

A third polymerized form of tubulin was induced by CP. While this polymer had the appearance of a continuous array of adjacent rings in thin section, negatively stained material indicated that relatively small clusters of rings were formed. Similar structures, at least superficially, have been reported by several groups using a wide variety of reaction conditions including vinca alkaloids (Bensch et al., 1969), high Mg^{2+} concentrations (Voter & Erickson, 1979), colchicine + vinblastine + glycerol (David-Pfeuty et al., 1979), and ATP + Mg^{2+} + glycerol (Zabrecky & Cole, 1980). Cold reversibility similar to that observed with CP has been reported only for the ATP-dependent polymer (Zabrecky & Cole, 1980).

We have also reported that several other organic anions stabilize the colchicine binding activity of tubulin, in particular δ-aminovalerate, 6-phosphogluconate, and glucose 6-phosphate (Hamel & Lin, 1980, 1981b). We have thus far not found conditions under which these compounds induce a cold-reversible polymerization reaction. The variety of polymerized forms of tubulin induced by organic acids, together with this apparent failure of several effective stabilizing agents to induce polymerization, may indicate that different interactions at the molecular level are involved in the stabilization and polymerization phenomena with different anions.

A variety of patterns of GTP hydrolysis were also observed. With CP, there was minimal nucleotide hydrolysis associated with polymerization, and the formation of the clusters of rings neither required GTP nor was inhibited by GDP. If these rings represent short protofilaments with reasonably normal longitudinal tubulin–tubulin interactions, our findings with CP support the suggestion that GTP hydrolysis results from the lateral contacts of tubulin molecules or protofilaments [cf. David-Pfeuty et al. (1977), Voter & Erickson (1979), and Weisenberg (1980)] rather than protofilament elongation.

In all cases in which microtubules or sheets of protofilaments were formed, GTP hydrolysis at least equaled tubulin polymerization, but the relative time of onset of polymerization and hydrolysis did not reflect polymer morphology. Simultaneous onset of the two reactions occurred with glutamate (sheets) and MAP's (microtubules), as well as with glycerol (microtubules). Significant lags were observed in the onset of polymerization relative to GTP hydrolysis with Mes (microtubules) and Pipes (sheets), as well as with glutarate, glucose 1-phosphate, and fructose 1,6-bisphosphate (sheets).

Although it is possible that the GTP hydrolysis induced by these anions is uncoupled from tubulin–tubulin interactions, two observations make this unlikely. First, the relationship of hydrolysis to tubulin concentration in Mes, Pipes, and glutamate (Figure 5), as well as with glucose 1-phosphate and fructose 1,6-bisphosphate (data not presented), is sigmoidal. This is predicted if tubulin–tubulin interactions are involved. Second, the initial rate of GTP hydrolysis at 3 mg/mL tubulin in Mes [0.7 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$] is identical with that with MAP's, despite the discordance in the onset of polymerization. Similarly, at 0.8 mg/mL tubulin, the initial rate in Pipes [0.4 mg min^{-1} mL^{-1} (mg of tubulin)$^{-1}$] resembles that in glutamate [0.5 nmol min^{-1} mL^{-1} (mg of tubulin)$^{-1}$]. These similar reaction rates suggest similar reaction mechanisms.

If GTP hydrolysis accompanies lateral tubulin–tubulin interactions, then the apparent uncoupling of hydrolysis and polymerization in Mes and Pipes probably derives from ongoing lateral interactions with deficient longitudinal (i.e., protofilament formation) interactions. Such lateral interactions without accompanying turbidity development may represent abortive nucleation reactions. The opposite, to an even greater extreme, occurs in CP: longitudinal without lateral interactions. Of the conditions we have examined, balanced reactions occur only with MAP's, glutamate, and glycerol, although morphologically different polymers are formed.

Acknowledgments

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Recycling of Cold-Stable Microtubules: Evidence That Cold Stability Is Due to Substoichiometric Polymer Blocks†

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ABSTRACT: A substantial subpopulation of mammalian brain crude extract microtubules is resistant to cold-temperature disassembly. We propose here that microtubules are rendered cold stable by rare substoichiometric blocks. Mild shearing of rat brain cold-stable microtubules makes them largely cold labile. In addition, cold-stable microtubules can be destabilized by exposure to low concentrations of calmodulin (5 μM) in the presence of calcium at 0 °C. Cold-disassembled microtubule protein, obtained from sheared or calmodulin-treated cold-stable preparations, re-forms a cold-stable subpopulation upon reassembly. These observations allow strategies for the recycling purification of cold-stable microtubules. Comparison of purified cold-labile and cold-stable material by gel electrophoresis shows enrichment for a few unique polypeptides, of 135, 70–82, and 56 kilodaltons, in the cold-stable preparation. The 64-kilodalton "switch protein", previously identified as uniquely dephosphorylated in cold-stable microtubules, is equally represented in recycled cold-stable and cold-labile microtubule preparations. Furthermore, when disassembled, cold-stable microtubule proteins are passed through a calmodulin affinity column on which the polypeptides characteristic of cold-stable microtubules are specifically retained, the breakthrough (unbound) material repolymerizes into cold-labile microtubules only. Based on the above data, a model is presented in which microtubules are rendered cold stable by the presence of substoichiometric, calmodulin-sensitive blocks that randomly reshuffle upon reassembly of cold-stable microtubules.

Microtubules in the cell cytoplasm and the mitotic apparatus, for the most part, rapidly depolymerize when exposed to cold temperature and to assembly-inhibiting drugs. A substantial subpopulation, however, remains resistant to cold temperature or to drugs that induce disassembly (Brinkley & Cartwright, 1975; Weber & Osborn, 1979; Salmon & Begg, 1980; Schliwa et al., 1981; Euteneuer & McIntosh, 1981).

When microtubules are polymerized in vitro from mammalian brain crude extract, cold-stable microtubules assemble in abundance (Grisham, 1976; Webb & Wilson, 1980). Comparing cold-stable and cold-labile populations, Webb & Wilson (1980) found no difference in either the protein composition or the isoelectric properties of tubulin. They also found that a "factor" could be separated from tubulin that conferred cold stability to cold-labile preparations.

We have recently demonstrated that cold-stable microtubules in rat brain crude extract are rapidly rendered cold labile upon addition of ATP (Margolis & Rauch, 1981). A 64-kilodalton polypeptide, designated the "switch protein" (Margolis & Rauch, 1981), is uniquely phosphorylated in the cold-labile microtubule population. This switch protein may play a role in regulating cold stability (Margolis & Rauch, 1981), although we have recently shown that STOP proteins that *release* from the polymer upon phosphorylation may be more centrally involved in cold-stability regulation (D. Job, C. T. Rauch, E. H. Fischer, and R. L. Margolis, unpublished results).

Purified cold-stable microtubules are insensitive to millimolar concentrations of free calcium, a condition that causes the rapid disassembly of cold-labile microtubules. However, addition of substoichiometric concentrations of Ca^{2+}-calmodulin causes the rapid disassembly of these same microtubules (Job et al., 1981).

Since low molar ratios of both the switch protein and calmodulin appear to influence microtubule assembly, it was suggested that cold stability is generated by the presence of certain substoichiometric blocks (Margolis & Rauch, 1981; Job et al., 1981). Evidence is presented here that such substoichiometric blockage indeed occurs. A recycling procedure for the purification of cold-stable microtubules was devised that allowed a comparison to be made of the protein compo-

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