Characterization of Rat Brain Crude Extract Microtubule Assembly: Correlation of Cold Stability with the Phosphorylation State of a Microtubule-Associated 64K Protein†

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ABSTRACT: We have conducted preliminary investigations into the control of microtubule assembly in rat brain crude extract supernatants. The rationale for these experiments is that microtubules interact with many proteins and are undoubtedly subject to physiological control mechanisms that are lost during tubulin purification. A more complete understanding of the cellular regulation of microtubules must include the physiology of these proteins. Assembly can be monitored in rat brain crude extract high-speed supernatants by measuring the increase in solution turbidity. We find that assembly is maximal in both rate and extent in the absence of added nucleotide. Increasing concentrations of either adenosine 5′-triphosphate (ATP) or guanosine 5′-triphosphate (GTP) inhibit both initiation and elongation of microtubules. GTP appears necessary for assembly and is apparently replenished from an intrinsic energy source during the time course of the assembly reaction. Inhibition of GTP production prevents microtubule assembly, and addition of exogenous GTP will reverse the blockage. Enzymatic removal of GTP at steady state causes a rapid depolymerization to the cold-stable microtubule level. Both GTP production and microtubule assembly display periodic oscillatory maxima. Cold-stable microtubules, which are always present in rat brain crude extract preparations, are rapidly made labile by addition of ATP. Analysis of proteins in cold-stable and cold-labile microtubule fractions shows changes in protein phosphorylation but not in the microtubule-associated protein composition. The tentative conclusion is that the state of phosphorylation of a 64K protein, designated the "switch protein", determines the cold stability or lability, and therefore the dimer association and dissociation rates, of crude extract microtubules.

The assembly mechanisms of purified mammalian brain microtubule protein have been well characterized, primarily by using porcine and bovine brain protein as source material [for reviews, see Snyder & McIntosh (1976), Scheele & Borisy (1979), and Timasheff and Grisham, (1980)]. Although some parameters of microtubule assembly in crude extracts from the brain have been studied by viscometry (Olmsted & Borisy, 1973; Asnes & Wilson, 1979), the emphasis of these studies has been to optimize assembly yields for the purification of microtubule protein by cycles of temperature-dependent assembly and disassembly.

In the purified brain microtubule system, there are a few major proteins associated with tubulin in the microtubule structure and a number of tightly associated enzymatic activities which affect microtubule assembly in vitro. Some enzymatic activities which affect microtubule behavior include an ATPase (Burns & Pollard, 1974; Gaskin et al., 1974b; White et al., 1980), a nucleoside diphosphate kinase (Berry & Shelanski, 1972; Jacobs et al., 1974), a cAMP-dependent protein kinase (Sloboda et al., 1975), and possibly an adenylate cyclase (Margolis & Wilson, 1980), and a phosphoprotein phosphatase (Coughlin et al., 1980). Associated structural proteins include two high molecular weight groups, MAP_1 and MAP_2,[1] both of which undergo phosphorylation reactions (Sloboda et al., 1975; Vallee, 1980), and the τ proteins (Weingarten et al., 1975; Cleveland et al., 1979), a series of proteins in the 55–70K-dalton size range. The associated structural proteins stimulate assembly (Weingarten et al., 1975), confer stability on the polymer structure (Murphy et al., 1977), and perhaps have microtubule cross-linking and dynein-like motility functions. Phosphorylation of MAP_2 also increases the microtubule treadmilling rate due to opposite-end assembly and disassembly behavior (a behavior apparently intrinsic to labile microtubules) (Margolis & Wilson, 1978, 1980; R. L. Margolis, C. T. Rauch, K. F. Sullivan, and L. Wilson, unpublished experiments).

All of these enzyme activities and protein associations have been found presumably because of their high affinity for the assembled microtubule. There are undoubtedly enzyme activities and associated proteins that have not been characterized because they do not quantitatively cosediment with microtubules, or because they are lost due to in vitro solution conditions or are lost to the discarded cold-stable microtubule fraction. For this reason, we have initiated a study of assembly characteristics of the crude extract material from rat brain.

Rat brain was chosen as a source because the material is conveniently available, and because preliminary studies showed that a simple turbidity assay accurately reflected microtubule assembly in this tissue. Bovine brain extracts, we found, do not clarify well enough upon centrifugation to be reliably assayed by turbidity measurement.

Rat brain crude extract microtubule assembly has supplied us with some surprising results. A high-speed supernatant, prepared under centrifugation conditions that hinder self-assembly of bovine or mouse brain tubulin, assembles rapidly and with high yield. Near optimal assembly was found to occur with no added nucleotide, and increasing concentrations of guanine nucleotide decreased both the rate and (to a lesser degree) the extent of assembly. In contrast, porcine and bovine brain crude extracts do not assemble in the absence of added nucleotide [except in the presence of glycerol (Shelanski et al., 1973)] and assemble optimally at 2.5 mM GTP (Borisy

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAPs, high molecular weight microtubule-associated proteins; MES, 2-(*N*-morpholino)ethanesulfonic acid; MEM buffer, 100 mM Mes, 1.0 mM EGTA, and 1.0 mM $MgSO_4$, pH 6.75; AMPPNP, adenylyl β,γ-imidodiphosphate; PCA, perchloric acid.

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et al., 1975; Asnes & Wilson, 1979).

There is an intrinsic energy source apparently available to drive the rat brain crude extract assembly. If guanine nucleotide levels are assayed during the crude extract assembly, we find that GTP levels rise and fall in an oscillatory manner despite the substantial hydrolysis that accompanies microtubule assembly. ATP, possibly acting by phosphorylating MAP proteins, inhibits initiation and elongation of microtubules in a concentration-dependent manner.

It is apparent that ATP has another role in regulating crude extract microtubule assembly. Recycled microtubules in vitro are rapidly disassembled when subjected to cold temperature (0-4 °C). There is, however, an abundant subpopulation of cold-stable microtubules present in the assembled brain crude extract material (Grisham, 1976; Webb & Wilson, 1980). We have found that these cold-stable microtubules are rapidly made labile by the addition of ATP and that a single substoichiometric microtubule-associated protein is uniquely phosphorylated when microtubules become cold labile. AMPPNP and adenosine γ-thiotriphosphate do not substitute for ATP in producing cold-labile microtubules from the cold-stable population.

Experimental Procedures

Materials. Reagents, buffers, and enzymes were purchased from Sigma Corp. Podophyllotoxin was purchased from Aldrich. Polyacrylamide gel materials were obtained from Bio-Rad and Radiolabeled nucleotides from New England Nuclear. Poly(ethyleneimine) chromatographic plates were purchased from Brinkmann Instruments. The scintillation fluor used was Aquasol-2, purchased from New England Nuclear.

Preparation of Rat Brain Crude Extract. Adult rats (inbred strain W/FU) were sacrificed by ethyl ether anesthesia followed by decapitation. Brains were quickly removed and added at 0 °C to MEM buffer composed as follows: (100 mM Mes, 1.0 mM EGTA, and 1.0 mM $MgSO_4$, pH 6.75).

Brains were homogenized in a ratio of 1.5 mL of MEM buffer per brain with a motor-driven Teflon and glass mortar and pestle (Bellco, 15 mL, loose fitting clearance) with three strokes at 2400 rpm. The brain homogenate was then centrifuged for 30 min (4 °C) at 200000xg in a Beckman L5-50 centrifuge using a Beckman 70.1 Ti rotor. The resulting supernatant was carefully removed and used for all subsequent analytical procedures. Total supernatant protein concentration is 16–20 mg/mL, of which approximately 22% is tubulin.

Turbidity Assay. Assembly of microtubule protein was monitored by the increase in turbidity that occurs during polymerization. It has been established for purified microtubule protein preparations that turbidity is linearly correlated with the mass concentration of polymer in solution (Gaskin et al., 1974a; MacNeal & Purich, 1978). We find, in this crude extract system, that more than 90% of the turbidity change observed is due to microtubule assembly.

Turbidity changes were monitored at 350 nm by using a Varian Cary 219 recording spectrophotometer equipped with a "cell programmer" for rapid successive timed assays of five separate samples. The sample chamber was maintained at a constant 30.0 ± 0.1 °C using a Neslab RTE-4 recirculating water bath.

Nucleotides and other materials added prior to or during assembly were first made up in stock solutions that caused a dilution of 1 part in 50 or less of the incubation mixture. All stock solutions were preadjusted to pH 6.75 in MEM buffer, except podophyllotoxin which was made as a millimolar stock in 100% dimethyl sulfoxide.

Assay of Nucleotide Levels during Assembly. Microtubules were assembled from rat brain extract in MEM buffer in the presence of 0.05 mM exogenous GTP. Either [α-^{32}P]GTP or [8-^{3}H]GTP (10 μCi/mL) was included during assembly. Samples (0.25 mL) were removed at time intervals and mixed with perchloric acid at 0 °C to a final PCA concentration of 7%. The precipitate was then removed by centrifugation in an Eppendorf 5412 centrifuge (5 min, maximum speed). The supernatant nucleotides were then separated according to the procedure of Penningroth et al. (1976). Briefly, the PCA supernatants were incubated at room temperature for 30 min with 10 mg each of activated charcoal, washed 3 times with 0.5 mL of water, then extracted from the charcoal in a 10% pyridine and 5% ethanol solvent, and finally chromatographed on Brinkmann poly(ethylenimine) plates in 1.2 M LiCl in the presence of known carrier nucleotides. The nucleotides were located under UV light and cut from the plates for scintillation counting.

Other Methods. Polyacrylamide slab gel electrophoresis and autoradiography were performed as described elsewhere (R. L. Margolis, C. T. Rauch, K. F. Sullivan, and L. Wilson, unpublished experiments) using conditions described by Sheir-Neiss et al. (1978). Electron microscopy specimens were prepared as previously described (Margolis & Wilson, 1978).

Results

Turbidity Assay. It has been shown that the turbidity of a purified brain microtubule protein solution increases as a linear function of the concentration of microtubule polymer under standard assembly conditions (Gaskin et al., 1974a; MacNeal & Purich, 1978). For the crude extract of bovine or porcine brain, however, aggregation of nonmicrotubule material upon warming can obscure microtubule assembly as assayed by turbidity. The standard assay for assembly in the crude extract of these microtubule protein sources has therefore been viscometry [see, e.g. Borisy et al. (1975) and Asnes & Wilson (1979)].

Rodent brain extracts (we have assayed rat, mouse, and hamster brains) are apparently quite different since they clarify well on centrifugation and can therefore be assayed directly for microtubule assembly by turbidity measurement. We have explored the parameters of rat brain crude extract assembly in detail since, as we will show, the rat brain has unique properties that make it an excellent source material for the study of the physiological regulation of microtubule assembly and treadmilling behavior.

The turbidity change that one measures upon warming the crude extract to 30 °C is nearly all due to microtubule assembly. In the presence of a high concentration of podophyllotoxin or colchicine, two highly potent microtubule assembly inhibitors (Wilson & Bryan, 1974), there is little change in turbidity (Figure 1A), representing no more than 10% of the turbidity change observed in the absence of the drug. By including a drug-containing control in each assembly assay and subtracting the control curve, one can routinely obtain the true assembly curve.

With increasing drug concentration, the concentration-dependent inhibition of microtubule assembly by colchicine and podophyllotoxin is reflected by an inhibition of solution turbidity change when assembly is monitored. A representative experimental run with podophyllotoxin, using drug concentrations in the micromolar range, is depicted in Figure 1A. The resulting apparent inhibition constants (K_i's) for podophyllotoxin and colchicine, calculated from the inhibition of turbidity change (Figure 1B), are 5.5×10^{-7} M and 5.0×10^{-7} M, respectively. Both values are in close agreement with values

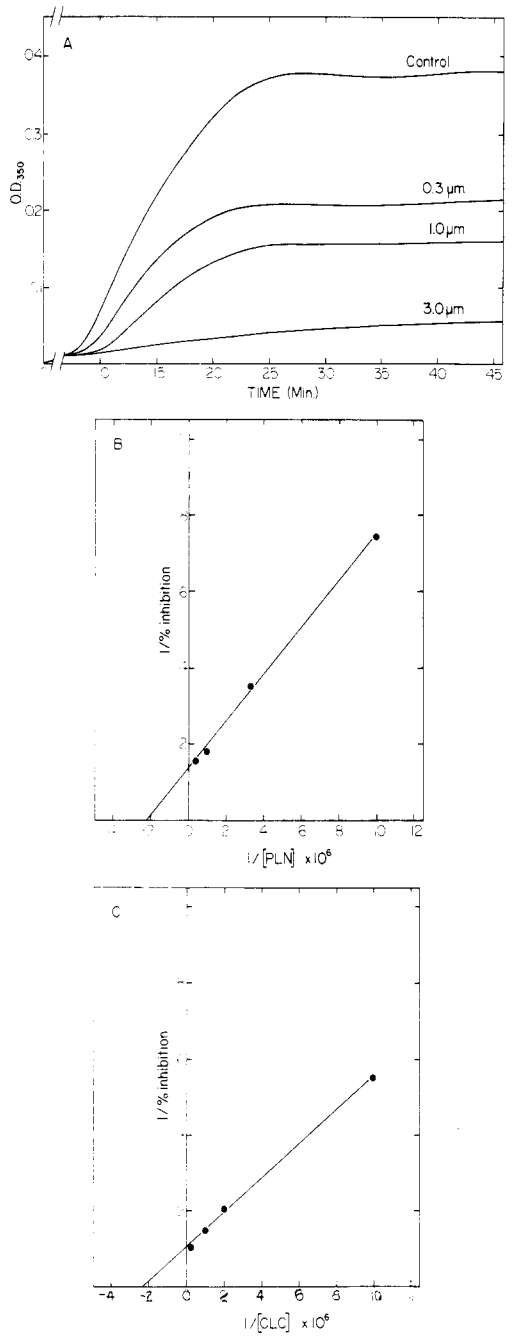

FIGURE 1: Drug inhibition of microtubule assembly. Rat brain crude extract supernatants were incubated at 30 °C in MEM buffer containing 0.1 mM GTP. Assembly was monitored turbidimetrically in a double-beam Cary 219 spectrophotometer against a buffer blank. Podophyllotoxin was added from appropriate stock solutions to the final concentrations indicated prior to warm-temperature incubation. (A) Effect of different indicated podophyllotoxin concentrations on assembly. (B) Apparent assembly rates obtained from the experiment depicted in (A) were used to determine the apparent inhibition constant (K_i) for podophyllotoxin. The K_i for this experimental run was 5.5 × 10^{-7} M. (C) A similar experiment performed with colchicine is represented. The K_i for colchicine was determined to be 5.0 × 10^{-7} M.

obtained for inhibition of assembly in vitro, both in brain crude extract material measured by viscometry and in purified preparations measured by turbidity (Wilson et al., 1976; Wilson & Margolis, 1978).

The apparent K_i's that we have obtained from turbidity measurements are expected values. High drug concentrations block more than 90% of the turbidity change. In other ways, such as the cold and drug sensitivity of the fully assembled polymer, the lack of turbidity increase in the absence of EGTA, and the colinearity of pelletable microtubule concentration with the change in optical density, the turbidity changes we are measuring are typical of in vitro microtubule assembly.

Turbidity accurately measures the extent of rat brain crude extract microtubule assembly, provided a slowly rising non-microtubule turbidity component (possibly NADH production) is subtracted from the result. This slowly rising component is determined during each turbidity experiment by inclusion of a sample containing 25 μM podophyllotoxin.

Effect of Guanine Nucleotide on Assembly. Porcine and bovine brain crude extract microtubule assembly has a GTP requirement. These microtubules will not assemble in the absence of added GTP, or of another nucleoside triphosphate that may promote assembly via nucleoside diphosphate kinase dependent phosphoryl group transfer to GDP. Assembly is optimal at 2.5 mM GTP (Borisy et al., 1975; Asnes & Wilson, 1979).

The difference of these systems from rat brain crude extract microtubule assembly is striking. Assembly of the rat brain crude extract microtubules is optimal when no nucleotide is added to the system (Figure 2). As the added nucleotide level is increased, polymer initiation and elongation both rapidly diminish. The effect on initiation can be distinguished from that on elongation by addition of a small amount of microtubule fragments as assembly seeds early in the incubation. When seeds are added, assembly is considerably more rapid at higher GTP concentrations (compare Figure 2B with 2A) than when they are not added. At 2.5 mM GTP, assembly is substantially suppressed.

The suppressing effect of guanine nucleotide on assembly is not strictly a property of GTP. Equivalent concentrations of GTP or GDP, added to the crude extract, suppress assembly equally (Figure 2C). As an explanation of this effect, it seems that crude extract enzymes quickly reduce GTP to mono and diphosphates at 0 °C, so that the nucleotide one adds is not the one present when assembly is initiated.

Requirement for GTP in Assembly. We have found that GTP is required for assembly in the rat brain crude extract system, despite the equivalence in effect of GTP and GDP on assembly, and the apparent inhibitory effect on assembly of increasing GTP concentrations. Following addition of GTP (0.05 mM, containing 10 μCi/mL [^{3}H]GTP) to the crude extract supernatant, rapid hydrolysis of the nucleotide to a nearly equal mixture of GDP and GMP occurs at 0–4 °C (time 0, Figure 3). Assay of nucleotide levels in such a preparation shows that, after warming to 30 °C, GTP is rapidly synthesized at the apparent expense of GMP, reaches a peak concentration after approximately 20 minutes at 30 °C, and then continues to deplete and reform in an oscillatory manner at about half its peak value (Figure 3). A long time course experiment (not shown) showed GTP levels remaining relatively constant to 2.5 hours (the last time point taken). The source for guanine nucleotide phosphorylation is as yet undetermined, but may be the reported large pool of phosphocreatine in the brain (Veech et al., 1979). The oscillatory nucleotide change is reflected in microtubule oscillations evident during assembly (Figure 2). Oscillations occur with an approximate 30-minute period in long time course experiments.

The formation of GTP appears critical to the assembly reaction. Sudden depletion of GTP from the crude extract after assembly is complete, using fructose 6-phosphate and phosphofructokinase to rapidly hydrolyze GTP (Margolis, 1981), produces a rapid disassembly of microtubules at a rate that is similar to that of podophyllotoxin-induced disassembly (Figure 4A). When nucleotide levels are assayed during the

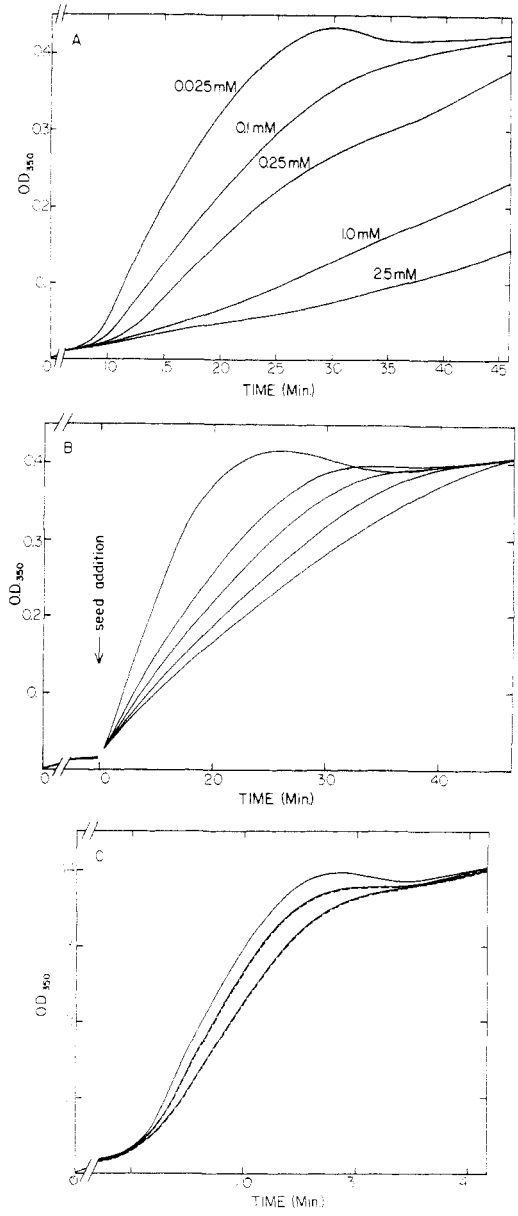

FIGURE 2: Effect of guanine nucleotide concentration on assembly. Rat brain crude extract supernatants were incubated at 30 °C in MEM buffer with varying concentrations of added GTP or GDP, as indicated. Assembly was monitored turbidimetrically. (A) Inhibitory effect of increasing exogenous GTP concentrations on assembly. The rate of elongation is substantially reduced by higher GTP concentrations. (B) Assembly of microtubules is monitored under conditions identical with those in (A) (same GTP concentrations) except that a 1:40 aliquot of microtubule fragment is added at the indicated time. Assembly is greatly accelerated by the addition of a small amount of seeds, indicating that the reduced elongation rate in (A) is largely due to a limited number of microtubule initiation events at higher GTP concentrations. Microtubule fragments were obtained from the same rat brain supernatant, assembled to steady state at 0.05 mM GTP and sheared by three passages through a 25-g needle prior to the assembly of the assayed material. Addition of fragments obtained from recycled bovine brain microtubules at steady state yielded an identical result. (C) Different concentrations of either GTP or GDP were added to rat brain extracts prior to assembly. Assembly was monitored turbidimetrically. The result indicates an identical inhibition of assembly at a given guanine nucleotide concentration, independent of whether it is the di- or triphosphate. Top line contains no added guanine nucleotide; second set of lines represents 0.05 mM guanine nucleotide; third (lowest) set represents 0.1 mM guanine nucleotide. GTP (—); GDP (---).

course of such an experiment, it is clear that GTP is rapidly hydrolyzed on fructose 6-phosphate addition (Figure 4B). In the experiment shown, GTP levels recover following fructose

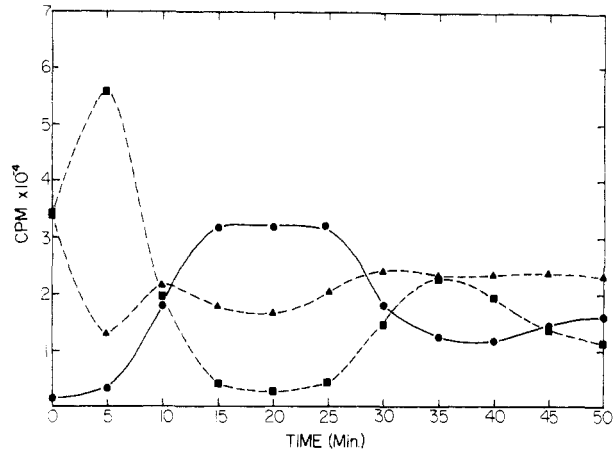

FIGURE 3: Fluctuations in guanine nucleotide levels during assembly. Rat brain crude extract supernatant was mixed, in MEM buffer at 0 °C, with 0.05 mM exogenous GTP containing 10 μCi/mL [8-^{3}H]GTP. After 10 min at 0 °C, microtubules were assembled by warming the solution to 30 °C. Samples were taken at the times indicated and mixed with perchloric acid on ice to a final PCA concentration of 7%. The samples were then extracted as described under Experimental Procedures and chromatographed on poly(ethylenimine) (PEI) thin-layer sheets. Each point represents a nucleotide spot from the PEI procedure. Both labeled and unlabeled GTP that were not added to the crude extract were more than 95% pure by chromatography. The figure depicts an assay of guanine nucleotide level during a typical assembly. The assembly reaction has plateaued at 30 min. The result shows that GTP is rapidly degraded to GMP and GDP at 0 °C prior to assembly, that GTP is formed at the expense of GMP during assembly, and that a relatively constant (but oscillating) GTP level is maintained after the initial burst. GTP (●); GDP (▲); GMP (■).

6-phosphate addition, but most often they do not. Microtubule reassembly onto available cold-stable microtubule seeds following fructose 6-phosphate addition is dependent on whether GTP levels recover following the block. If either 1.0 mM AMPPNP or 10 mM fructose 6-phosphate is present when crude extract assembly is initiated, no assembly occurs (Figure 4A). In accord with this result, we find that GTP is not produced in the extract in the presence of these inhibitors (result with AMPPNP is shown in Figure 4C). The enzyme site of blockage by AMPPNP is presently unknown, although it is a potent glycolysis inhibitor (K_i = 20 μM) (Hill & Hammes, 1975). Phosphofructokinase allosteric inhibitors (and therefore glycolysis inhibitors) citrate and 2,3-diphosphoglycerate (Krzanowski & Matschinsky, 1969) also have been found to simultaneously deplete GTP and suppress microtubule assembly. Based on all the present evidence, we believe GTP is a requirement for both initiating and sustaining the cold-labile microtubule assembled state.

Regulation of Assembly by ATP. Increasing concentrations of ATP, added to the rat brain crude extract supernatant, suppress assembly (Figure 5A). The time of initiation of assembly and, to a lesser extent, the rate of assembly are both affected by increasing nucleotide concentrations. As was the case with guanine nucleotide induced suppression, addition of a 1:40 aliquot of short microtubule fragments to act as assembly seeds somewhat ameliorates the nucleotide-induced blockage (Figure 5B).

The effect of ATP is probably at least 2-fold. In addition to the suppression of assembly described above, ATP causes the rapid loss of cold stability in crude extract microtubules. Normally, microtubules that assemble in the crude extract of brain tissue contain a subpopulation that is highly resistant to cold temperature induced disassembly (Grisham, 1976). This subpopulation of microtubules is, further, resistant to drug-induced disassembly but can be depolymerized by mil-

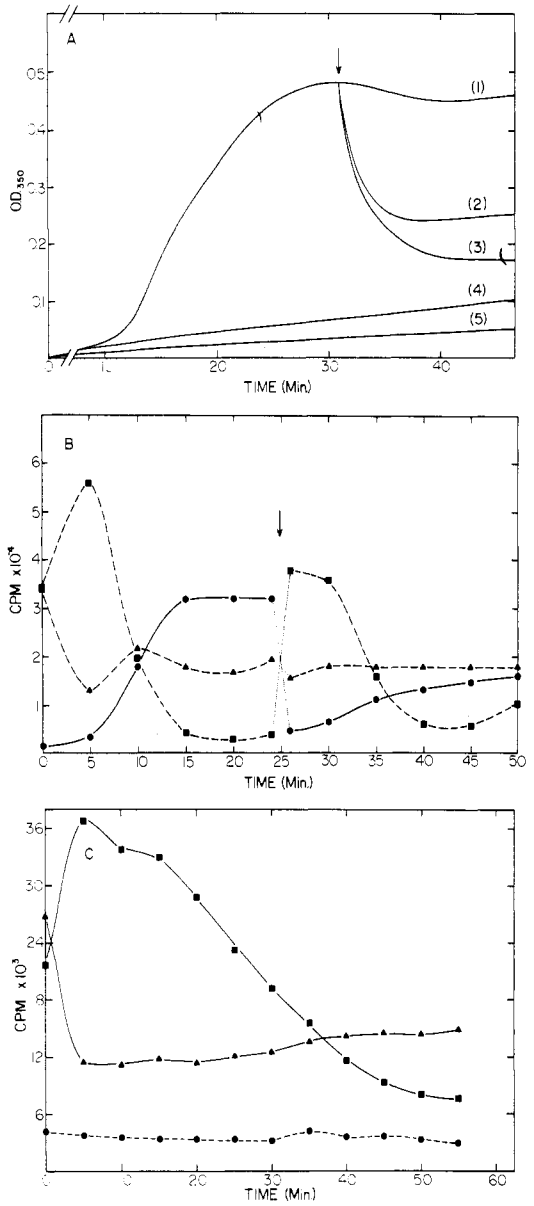

FIGURE 4: Effect of metabolic intermediates on microtubule assembly. Rat brain crude extract supernatants were incubated at 30 °C in MEM buffer containing 0.05 mM GTP, and assembly was monitored turbidimetrically. (A) Fructose 6-phosphate (10 mM) was either added prior to 30 °C incubation (4) or added to the solution once a full assembly plateau had been obtained (2). The result shows fructose 6-phosphate substantially inhibits assembly or causes depolymerization of fully assembled microtubules with the same apparent rate, but to somewhat less an extent than obtained with podophyllotoxin (3). The final plateau is apparently due largely to the cold-stable microtubule population. AMPPNP (1.0 mM), added prior to assembly, totally inhibits assembly (5). Control (no additions) is also shown (1). (B) Assay of guanine nucleotide levels after fructose 6-phosphate addition; performed as described in Figure 3. Fructose 6-phosphate (10 mM) was added during assembly where indicated by an arrow. The result shows that GMP is rapidly produced in fructose 6-phosphate at the expense of GTP, and, thereafter, GTP levels recover. Sometimes we find a recovery of GTP following fructose 6-phosphate addition (as here), and sometimes no recovery is evident. GTP (●); GDP (▲); GMP (■). (C) Assay of guanine nucleotide levels during crude extract incubation at 30 °C following 1.0 mM AMPPNP addition at time 0. AMPPNP totally suppresses GTP production and also totally suppresses assembly (Figure 4A). GTP (●); GDP (▲); GMP (■).

limolar calcium (Webb & Wilson, 1980).

In Figure 6, we show the cold and drug resistance of these microtubules (confirmed by electron microscopy to be present). If millimolar ATP is added to cold-resistant drug-blocked microtubules, the microtubules disassemble completely within

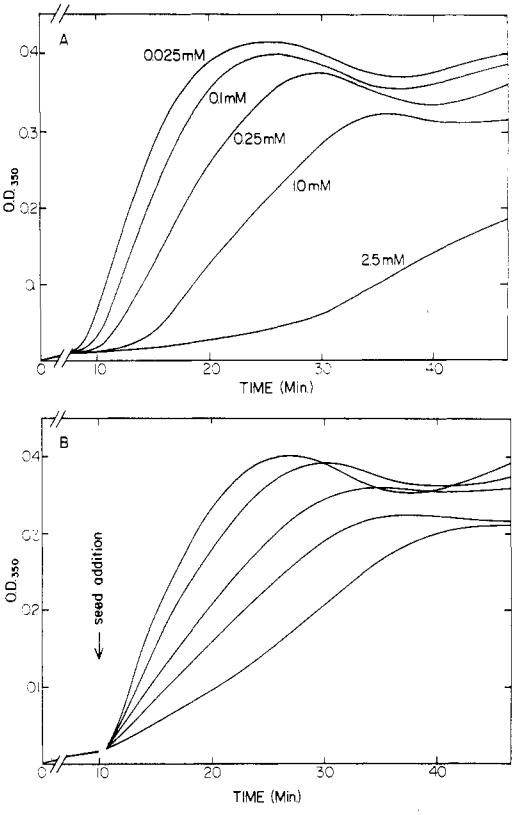

FIGURE 5: Effect of adenine nucleotide concentration on assembly. Rat brain crude supernatants were incubated at 30 °C in MEM buffer containing 0.05 mM GTP. Varying concentrations of ATP, as indicated, were included during incubation. (A) Inhibitory effect of increasing exogenous ATP concentrations on assembly. (B) Assembly of microtubules under conditions identical with those in (A) (same ATP concentrations) except that a 1:40 aliquot of microtubule fragments was added at the indicated time. Microtubule fragments were prepared as described in the legend to Figure 2.

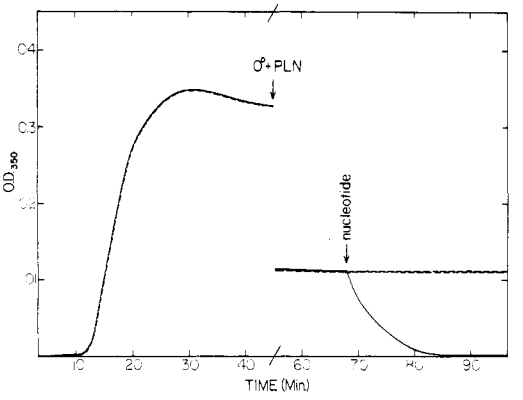

FIGURE 6: The effect of ATP on cold-stable microtubules. Rat brain crude extract supernatant was assembled at 30 °C in MEM buffer containing 0.05 mM GTP. Once an assembly plateau had been obtained, microtubules were exposed to 25 μM podophyllotoxin and chilled to 0 °C for 10 min. When rewarmed, they were at their cold-stable level. Cold-stable microtubules were then exposed to 1.0 mM ATP, 1.0 mM AMPPNP, or 1.0 mM adenosine γ-thiotriphosphate. Only ATP caused the rapid disassembly of the cold-stable population. ATP (—); AMPPNP and adenosine γ-thiotriphosphate (- - -).

5 minutes (Figure 6). Neither AMPPNP nor adenosine γ-thiotriphosphate will substitute for ATP in inducing this state of increased lability.

Polyacrylamide gels of the cold-stable vs. total crude extract microtubules, isolated by centrifugation through sucrose, show no difference in protein composition (Figure 7), as reported previously by Webb & Wilson (1980). However, exposure of the microtubules to [γ-^{32}P]ATP before isolation (result is

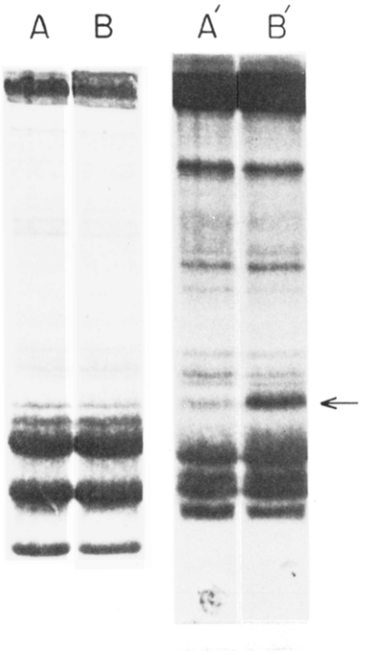

FIGURE 7: Comparison of protein phosphorylation in cold-stable and cold-labile microtubules. Rat brain crude extract microtubules were assembled at 30 °C to plateau in MEM buffer containing 0.05 mM GTP and then exposed to 50 μCi/mL [γ-^{32}P]ATP for, in this instance, 15 min at 30 °C prior to isolation by centrifugation through 50% sucrose. Total assembled microtubule protein, collected through sucrose cushions, is shown in lane A, stained with Coomassie blue. The cold-stable subpopulation, chilled to 0 °C for 10 min at the end of the 30 °C incubation, rewarmed in 25 μM podophyllotoxin, and then isolated by centrifugation, is shown in lane B. The lowest densely stained band is actin; the two higher heavy bands are α- and β-tubulin. Bands at the top of the gel include the microtubule-associated proteins (MAPs). The autoradiograph of this gel is on the right (X-ray film exposure of 1 week). Lane A′ is the autoradiograph of lane A, and B′ is that of B. The arrow indicates the 64K protein band that is phosphorylated in the total microtubule population but not phosphorylated in the cold-stable population. All other ^{32}P bands are comparable between the two preparations. Time of exposure to the [^{32}P]ATP was varied in other experiments between 5 min and 0.5 h, with the same result. The polyacrylamide gel procedure used here is that of Sheir-Neiss et al. (1978). Switch-protein molecular weight was determined by comparison to Bio-Rad high molecular weight protein standards on Sheir-Neiss et al. (1978) slab gels.

the same with label exposure times varied between 5 and 30 min) reveals a protein of approximately 64K daltons (Figure 7, arrow) that is phosphorylated in the total microtubule preparation but is uniquely dephosphorylated in the cold-stable subpopulation. This protein meets the criteria of a factor that would cause microtubules to be stable or labile (in other words, kinetically active or inactive) due to its state of phosphorylation. We tentatively refer to this protein as "switch protein" for its possible role in turning on and off the kinetic function of microtubules.

Discussion

Crude Extract Assembly Properties. The rat brain crude extract displays remarkable microtubule assembly properties that make it an excellent source material for in vitro studies under circumstances close to the intracellular condition. Two such properties are the clarity of supernatant solutions from the extract and the lack of extrinsic nucleotide requirement for assembly. The clear supernatant solutions allow quantitative measurement of assembly by turbidity assay, giving one experimental flexibility not permitted by other mammalian brain crude extract preparations. Lack of extrinsic nucleotide requirement is important since it potentially allows one to perform steady-state kinetic experiments by labeled GTP pulse and chase (R. L. Margolis and C. T. Rauch, unpublished experiments). Another unique property of the rat brain extract is the ability of the microtubules to assemble in high speed centrifugation supernatants. Beef and porcine brain extracts have lost essential initiating seeds under these conditions and will not assemble.

It is apparent that nucleotide need not be added to the extract to obtain assembly because there is an intrinsic and very active nucleoside triphosphate regenerating system. Additional nucleotide, either ATP or GTP, suppresses both initiation and elongation of microtubules. Since both GTP and ATP are rapidly degraded to the nucleoside mono- and diphosphates at 0 °C prior to assembly (Figure 3; R. L. Margolis and C. T. Rauch, unpublished experiments), the inhibition may simply represent the decreasing ability of the extract to produce enough GTP to drive assembly at high nucleotide concentrations. This possibility remains to be tested. Other brain crude extracts, porcine and bovine, require an optimum of 2.5 mM GTP for assembly, and assembly will not occur under our buffer conditions in the absence of added guanine nucleotide.

There are a few interesting features to note concerning guanine nucleotide production in the crude extract. First, in this system, it is evident that GTP rapidly degrades at 0 °C to GMP and GDP. Although we have not assayed guanine nucleotide levels in other brain preparations, a similar reaction in bovine or porcine crude extracts could greatly alter exogenous nucleotide requirements for assembly. Second, the GTP and GMP levels clearly reciprocally oscillate (Figures 3 and 4) while GDP levels remain relatively stable. Since the expected pathway of GTP production is phosphocreatine utilization to form ATP, and ATP phosphotransfer first to GMP (by adenylate kinase) and then to GDP (by nucleoside diphosphate kinase) (Lehninger, 1970), the reciprocal exchange between GTP and GMP suggests that another pathway may be operating here. Further work to carefully follow the different nucleotides is in order. Third, the oscillatory nature of nucleotide production indicates that the metabolic controls, as for oscillations of the glycolytic cycle (Boiteau et al., 1975), may be multiple and complex. If GTP production is controlled by creatine kinase in this system, then the root cause of the oscillation may be of interest. However, creatine kinase has no known feedback inhibition or activation loops; thus, its direct participation in the creation of oscillation is doubtful. More likely its ADP substrate levels oscillate due to intact glycolytic pathway feedback loops, and thus the gate of ATP production from phosphocreatine opens and closes. We have, in fact, observed that ADP and ATP undergo periodic oscillations similar in nature to the guanine nucleotide oscillations reported here (R. L. Margolis and C. T. Rauch, unpublished experiments). The fact that assembled microtubules have reproducible oscillatory maxima that coincide in time with GTP peaks indicates how sensitive microtubules are to GTP level changes. Similar oscillations in vivo could obviously play some role in various biological oscillations, particularly of the brain.

Compounds that deplete GTP or inhibit its production, including AMPPNP, fructose 6-phosphate, citrate, and 2,3-diphosphoglycerate, also inhibit microtubule assembly. Their assembly blockage is coincident with a lack of GTP in the extract and is rapidly reversed by the addition of GTP. Fructose 6-phosphate, in the presence of phosphofructokinase, rapidly hydrolyzes GTP, blocks steady-state microtubule treadmilling in vitro, and, in a purified beef brain microtubule

preparation, creates a nonequilibrium metastable state (Margolis, 1981). In the crude extract of rat brain, fructose 6-phosphate, by rapidly depleting GTP, causes the rapid disassembly of microtubules, leaving largely the drug- and cold-stable subpopulation. The metastable state produced in purified beef brain microtubules by GDP (Margolis, 1981) also appears to be present in the crude extract preparation under conditions where the microtubules remain cold labile (data not shown). In neither case (crude extract or purified) is a net assembly of GDP–tubulin observable. We find no evidence thus far, under two very disparate assembly conditions, for GDP–tubulin assembly when GTP-driven assembly is suppressed.

It has been proposed that microtubules emanating from the perinuclear region in fibroblast cells have blocked net disassembly ends and are preserved in an assembled state by continuous GTP hydrolysis at the net assembly end (Kirschner, 1980). The rapid disassembly of rat brain crude extract microtubules in the absence of GTP may be supportive of this suggestion. However, we do not know if loss occurs primarily at the net assembly or at the net disassembly end of the microtubule under these circumstances. Preliminary evidence suggests, either additionally or to the contrary, that a very rapid treadmilling of subunits accounts for a substantial portion of the rapid disassembly observed in GDP or podophyllotoxin (R. L. Margolis and C. T. Rauch, unpublished experiments).

Cold-Stable Microtubules: Switch Protein Dependent Kinetic Stabilization. Cold-stable microtubules represent a substantial proportion of the total microtubules that assemble in a mammalian brain extract (Grisham, 1976; Webb & Wilson, 1980). If crude extract, and intracellular, microtubules can maintain rapid equilibria dependent on GTP hydrolysis, it is reasonable to suspect that the cell would contain the machinery to conserve energy in proper circumstances by stabilizing preexisting microtubules.

The data we have presented here demonstrate a rapid ATP-dependent labilization of cold-stable (and drug-stable) microtubules. In the presence of podophyllotoxin, for instance, ATP causes the rapid and total depolymerization of previously stable microtubules.

A specific microtubule-associated protein phosphorylation is the most likely cause of the ATP-dependent switch from cold stability to cold lability. This microtubule protein should be phosphorylated when the polymer is cold labile and dephosphorylated when it is cold stable. Gel electrophoresis autoradiography reveals one protein that meets these criteria, a 64K-dalton phosphoprotein that we designate the switch protein. It is in the molecular weight region of previously identified microtubule-associated τ proteins (Cleveland et al., 1979), but whether it is a τ protein is not now known. If the switch protein is directly associated with cold stability and it is a τ protein, then it must be phosphorylated when present in purified cold-labile microtubule preparations; otherwise, the microtubules would be constitutively cold stable.

We have recently confirmed switch-protein presence in crude extract microtubules of another mammalian (bovine) brain (D. Job and R. L. Margolis, unpublished experiments) and are working to isolate and characterize this protein. It is clear from gel electrophoresis results that recycled bovine brain microtubules prepared in our lab, which are constitutively cold labile, lack the switch protein.

This putative switch protein can operate at concentrations substoichiometric to that of tubulin, since a disassembly block will occur whenever the phosphate-free protein occupies the net assembly or net disassembly end of the microtubule. It is important to note that a substoichiometric block of cold-temperature disassembly by the switch protein is consistent with the reported completely end-dependent disassembly of recycled bovine brain microtubules at cold temperature (Karr et al., 1980). The determinant of whether a microtubule is in an active equilibrium or a stable state may therefore depend on the physiological state of protein kinase machinery in the near locale of a microtubule's assembly or disassembly end. It is further conceivable that certain cells maintain microtubules in a constitutive active equilibrium by failure to synthesize a switch-protein-like molecule.

The mitotic apparatus of some cells maintains cold- and drug-stable microtubules (kinetochore to pole microtubules) adjacent to cold-labile (interpolar) microtubules (Brinkley & Cartwright, 1975). Whether switch-protein and kinase-dependent mechanisms operate here to maintain two distinct populations of microtubules is unknown, and further, the detailed mechanism that might create this situation is difficult to conceptualize. Nonetheless, this possibility must now be considered carefully.

It is important to emphasize that while a correlation of 64K protein phosphorylation with the in vitro cold-stable state exists, this protein may be only part of a more complex system or may not be causally involved in cold stabilization at all. Further work to purify the components and reconstitute the system, currently under way in our laboratory, will resolve this issue.

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Nucleophilic Modification of Human Complement Protein C3: Correlation of Conformational Changes with Acquisition of C3b-like Functional Properties†

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ABSTRACT: Inactivation of C3 by enzymatic cleavage, nucleophilic addition, or slow freezing and thawing resulted in the acquisition of similar end-state conformations as judged by near-UV circular dichroism. Although inactivation by the two nonenzymatic processes involves no peptide bond scission, the inactivated C3 resembled C3b in that it possessed a free sulfhydryl group not present in the native protein and an increased surface hydrophobicity as evidenced by enhanced binding of the fluorophore 8-anilino-1-naphthalenesulfonate (ANS). The C3b-like functional properties of modified C3 [Pangburn, M. K., & Müller-Eberhard, H. J. (1980) *J. Exp. Med. 152*, 1102–1114] may thus be understood in terms of the similarity of its conformation to that of C3b. The rate of the conformational change following proteolytic cleavage was fast and appeared to be limited by the rate of the enzymatic reaction. In contrast, the rate of conformational change following addition of methylamine was slow and rate limited by the conformational rearrangement itself, not by the chemical modification. A kinetic analysis of the changes in circular dichroism and ANS fluorescence enhancement suggested that the nucleophilic addition was spectroscopically undetectable and was followed by a minimally biphasic, spectroscopically demonstrable conformational rearrangement. The appearance of C3b-like functional activity in nucleophile-modified C3 largely parallels the time course of the spectroscopically detectable conformational change but is distinctly slower than the rate at which hemolytic activity is lost. While fully transconformed methylamine-inactivated C3 can bind factor B and is susceptible to cleavage by C3b inactivator and its cofactor β1H, this cleavage occurs at a substantially slower rate than the equivalent process in C3b. The implications of these findings in terms of the mechanism through which the alternative pathway of complement is initiated are discussed.

C3 is a highly versatile serum protein which plays a pivotal role in host defense and the inflammatory functions of complement. As such, C3 is the precursor of several physiologically occurring fragments which have different biological activities (Müller-Eberhard & Schreiber, 1980). The protein has a molecular weight of 187 500 (Tack & Prahl, 1976) and is composed of two polypeptide chains, the molecular weights of which are approximately 115 000 (α chain) and 75 000 (β chain). Upon complement activation, the enzyme C3 convertase cleaves peptide bond 77 of the α chain of C3, thereby producing the fragments C3a (M_r 9000) and C3b (M_r 178 500) (Hugli & Müller-Eberhard, 1978). C3a constitutes one of the three anaphylatoxins of the complement system, and C3b fulfills various functions in the cytolytic and opsonic com-

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