Isolation from Bovine Brain of a Superstable Microtubule Subpopulation with Microtubule Seeding Activity†

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ABSTRACT: Cold-stable microtubule protein isolated from beef brain is capable of seeding microtubule assembly under conditions that prevent the initiation of self-assembly of cold-labile microtubules. We have developed a quantitative assay for the determination of seeding activity. Using this assay, we find that seeding activity is apparently due to microtubule fragments that resist −80 °C, a condition that causes the depolymerization of cold-stable microtubules ("cold stability" is defined as resistance to 0 °C disassembly), but rapidly depolymerize when exposed to 3.0 mM free calcium, to micromolar Ca^{2+}–calmodulin, or to 0.2 M NaCl at 4 °C. After salt treatment, seeding activity is permanently lost although microtubule cold stability is retained through further assembly cycles. Similarly, after sedimentation of microtubule seeds the supernatant protein assembles into cold-stable microtubules, which are permanently devoid of seeding activity. By contrast, seeding activity can be recovered by recycling of supernatant protein from preparations exposed to 3.0 mM calcium or to Ca^{2+}–calmodulin prior to centrifugation, indicating the solubilization of an active component (designated "preseeds") under these conditions. Polyacrylamide gels show some differences in polypeptides between seeding and nonseeding cold-stable microtubule preparations. Approximately 35% of the microtubule population assembled from beef brain crude extract is cold stable, while approximately 2% constitutes −80 °C resistant seeds. The formation of seeds from seed-forming subunits (preseeds) occurs rapidly, is apparently a cooperative phenomenon, and occurs on preexisting microtubules under either assembly initiating or steady-state conditions.

Microtubules perform a wide variety of functions in cells. They are, for example, centrally involved in mitosis and other motility phenomena such as the transport of subcellular organelles or changes in cell shape [for reviews, see Dustin (1978) and Roberts & Hyams (1979)]. Cytoplasmic microtubules are for the most part highly labile and are in constant equilibrium with their tubulin subunits. The study of labile microtubules in vitro has established many of the molecular requirements for their assembly and disassembly [for a review, see Timasheff & Grisham (1980)]. Further, it has been shown that they have the intrinsic property of net unidirectional subunit flow at steady state, or treadmilling (Margolis & Wilson, 1978, 1981).

Treadmilling may be important for microtubules to function as cellular machinery, since it makes the polymers into machines with unidirectional flow properties. It is clear, however, that free treadmilling alone is not sufficient to describe microtubule behavior as machines. Effective work must require translocation of polymers past fixed objects or of attached molecules on stable microtubules. The physiological regulation of subunit addition and loss that might allow coordination with other mechanistic events is therefore of great importance to understand.

We have been studying cold-stable microtubules, a subpopulation purified from brain tissue that exhibits profound response to regulatory stimuli, including Ca^{2+}–calmodulin and protein kinase. These microtubules are resistant to cold-temperature (0 °C), calcium-induced or drug-induced disassembly (Grisham, 1976; Webb & Wilson, 1980; Margolis & Rauch, 1981) but become highly labile when reacted with calmodulin (Job et al., 1981; Pirollet et al., 1983) or ATP (Margolis & Rauch, 1981; Pirollet et al., 1983; Job et al., 1983).

A small group of cold-stable microtubule associated polypeptides, designated STOPs (stable tubule only polypeptides), appears to be intimately associated with cold-stabilizing activity (Job et al., 1982). This result, and the ability to induce cold-temperature disassembly of stable microtubules by mild shearing (Job et al., 1982), requires rare and random blocks that prevent endwise depolymerization of the polymer.

Disassembly was thus shown to be a highly regulated phenomenon in these polymers. In the course of these studies, we also observed that assembly of cold-stable microtubules initiated with no time lag [for example, see Pirollet et al. (1983), Figure 2]. This result suggested that, in contrast to cold-labile microtubule protein, the cold-stable fraction contains preformed assembly seeds.

Here we report that regulation of assembly by seeding is created by "superstable" microtubule fragments contained as a subpopulation among cold-stable microtubules. These superstable polymers (seeds) are distinguished from previously described cold-stable microtubules by their resistance to −80 °C or sonication. They may, however, be disassembled by calmodulin and will subsequently re-form. The capacity to create superstable polymers is separable from cold stability since cold-stable microtubules may be isolated that have no capacity to form these stable assembly seeds.

While cold stability appears to be due to highly substoichiometric STOPs, seeding activity may be due to the cooperative interaction of unique, relatively abundant polypeptides found associated with the tubulin on microtubule seeds.

We have yet to determine if seeding is a phenomenon entirely separate from STOP-related disassembly inhibition or if it represents the interaction of additional polypeptides with the STOP polypeptides to create superstable assembly starts.

As an important ancillary to these results, we report here the first bulk isolation of cold-stable microtubules from a common large mammal brain source, that of beef (we have

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0006-2960/84/0423-3025$01.50/0

previously reported on purification from rat and sheep brains). Since superstable microtubules are rare (approximately 2% of the total microtubules that assemble in the crude extract), such large animal isolation procedures are essential to perform meaningful biochemistry on this subfraction.

We have established a nomenclature for the materials under study in order to facilitate their discussion. "Seeds" will refer to the superstable microtubule fragments that are capable of acting as assembly seeds for polymer elongation. "Preseeds" will refer to seed-forming subunits, disassembled from seeds, which have the capability of reassembling into superstable microtubule fragments.

Materials and Methods

Materials. All materials used are the same as previously reported (Job et al., 1983), except that all nucleotides are purchased from Boehringer-Mannheim.

Isolation of Cold-Stable Microtubule Protein from Beef Brain. Procedures for the isolation of cold-stable microtubule protein from beef brain are similar to those previously published for sheep brain (Pirollet et al., 1983) with modifications as described below. We find that this method, successfully applied to one large animal brain source, may be applied to material from other sources.

A brief summary of the general procedure follows. Beef brains are kept at 4 °C and must be processed in the laboratory within 1 h after death. Homogenization is carried out by using a rotary blade (Waring) homogenizer in 1.5 mL of buffer/1.0 g of brain matter. The homogenization time is 30 s at a low setting.

The buffer used for homogenization is similar to that used for experimental work [MME, composed to 100 mM 4-morpholineethanesulfonic acid (Mes), 1 mM $MgCl_2$, and 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) at pH 6.75] but additionally contains 2 mM $CaCl_2$ and 4 mM EGTA, added successively [see Pirollet et al. (1983)]. The homogenized material is centrifuged for 30 min, 120000*g* (average), at 4 °C. Supernatant fractions are collected and assembled for 1 h at 30 °C in MME buffer containing 1 mM GTP. Unlike sheep brain extract, beef brain crude extract assembly cannot be monitored by turbidity increase (due to unacceptable background "noise"). Following assembly, the protein is chilled to 6 °C for at least 10 min and then layered on 50% sucrose in MME buffer and centrifuged in a fixed-angle rotor for 1.5 h at 120000*g* (average) at 25 °C.

Pellets are resuspended in MME buffer to a final concentration of 10 mg/mL, and the protein suspension is frozen in a −80 °C freezer, thawed again, and centrifuged at 40000*g* for 0.5 h at 4 °C. The final supernatant fraction is refrozen for later use.

Isolation of Cold-Labile Microtubule Protein. Cold-labile microtubule protein from beef brain is isolated by three cycles of assembly and disassembly according to published procedures (Margolis & Wilson, 1978; Asnes & Wilson, 1979) with the following modification. For the third assembly cycle, protein is resuspended from pelleted microtubules and centrifuged in MME buffer for 30 min, 120000*g* (average), at 4 °C. The supernatant fraction is reassembled in 2 mM GTP at 30 °C for 45 min, layered on 50% sucrose in MME buffer, and centrifuged 2 h in a fixed-angle rotor (120000*g* average, 30 °C). Pellets are stored at −80 °C for later use.

Other Procedures. Other procedures, including turbidity measurement, electron microscopy, and polyacrylamide gel electrophoresis, are performed as previously reported (Job et al., 1983). Protein concentrations are routinely determined by OD_{280} measurement, where 1.0 mg/mL microtubule protein equals 1.0 OD unit in the absence of nucleotide.

Results

Demonstration of Assembly Seeding Activity. Recycled microtubule protein fails to assemble when GDP exceeds the GTP concentration by 10-fold. Under these restricted assembly conditions, initiation of assembly is suppressed while elongation from microtubule starts or seeds proceeds normally.

Cold-stable microtubule protein, subjected to the same stringent conditions, assembles in 10-fold excess GDP (Figure 1A). This protein population, derived from purified cold-stable microtubules by disassembly at −80 °C, must contain an assembly-initiating seed absent from cold-labile preparations. A mixture of the two protein populations, cold stable and cold labile, at different concentrations, confirms that the activity correlates in a linear manner with the relative concentration of cold-stable protein (Figure 1B,C).

Disturbance of Assembly Seeding Activity. Having established that the microtubule seeding activity of the cold-stable microtubule subpopulation resists −80 °C, we found by electron microscopy that microtubule fragments remain in the −80 °C resistant material. Naturally, such fragments are prime candidates for a direct role in the observed seeding phenomenon. We have established previously that cold-stable microtubules disassemble in the presence of millimolar calcium and cold temperature combined or when exposed to micromolar Ca^{2+}–calmodulin (Job et al., 1981). Exposure of the −80 °C treated protein to increasing calcium concentrations for 1 h produces an equivalently diminished seeding response in microtubules subsequently assembled in high GDP (Figure 2A). The effect of calcium is on seeding of assembly, since shearing of a weakly assembling preparation (exposed to 1 mM free calcium for 0.5 h prior to assembly) restores an excellent assembly response. Under these conditions, numerous seeds are introduced by fragmenting the few preexisting microtubules (Figure 2B).

A loss of assembly seeds is also observed following exposure to calmodulin for a brief period of time. Ten-minute exposure to Ca^{2+}–calmodulin yields a substantial concentration-dependent suppression of seeded assembly in the next assembly cycle (Figure 3A). At a calmodulin concentration of 5 μM, seeding activity is totally abolished by such a 10-min incubation. In a similar manner, exposure to increasing salt in a range up to 0.2 M also suppresses seeded assembly in a concentration-dependent manner (Figure 3B).

Remarkably, sonication of assembly seeds for 45 s has no effect on seeding activity, nor does it visibly alter superstable microtubules. In Figure 4, we show microtubules that remain after −80 °C treatment and subsequent sonication. Cold-stable microtubules with no assembly seeding activity are totally depolymerized by either of these treatments.

Recovery of Seeding Activity. Exposure to millimolar calcium, to micromolar calmodulin, or to moderate salt concentrations, all at 0 °C, causes the disappearance of seeding activity. Assembly seeds may re-form in the next assembly cycle, however, and lead to the reappearance of seeded assembly in the following (second) assembly cycle. After the first recovery cycle, a −80 °C disassembly step destroys all but the superstable assembly seeds. After this treatment, we find assembly seeding activity has been restored to calmodulin-treated microtubules but not to the salt-treated samples (Figure 5). Interestingly, both salt- and calmodulin-treated samples, assembled with GTP, are similarly cold stable (Figure 5). Thus, salt treatment selectively destroys assembly seeding activity and causes no change in cold stability.

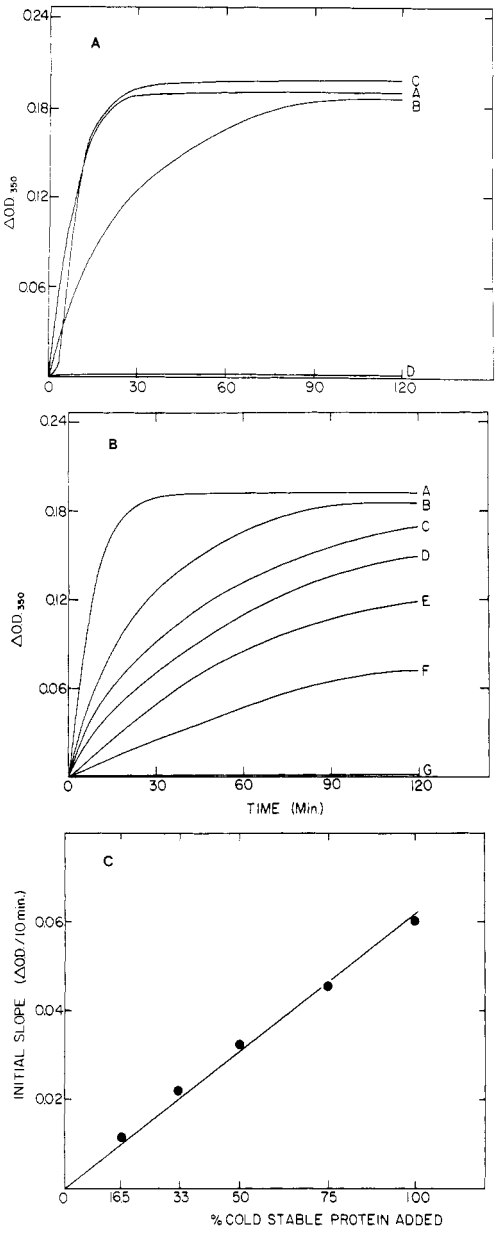

FIGURE 1: Assay of microtubule seeding activity. (A) Comparison of cold-stable and cold-labile microtubule protein for ability to seed assembly in high GDP. Lines A and B show cold-stable microtubule assembly in (line A) 0.5 mM GTP or (line B) 0.5 mM GDP, 0.05 mM GTP, and 1.0 mM AMPPNP. Lines C and D show cold-labile microtubule assembly under the same conditions. Cold-labile and cold-stable microtubule preparations are three-cycle and two-cycle purified, respectively, as described under Materials and Methods. Assembly is initiated at warm temperature (30 °C) by the addition of GTP after the adjustment of base lines. There is no base-line shift upon addition of nucleotide. Protein concentration is 1.2 mg/mL. The assay measures turbidity development at 350 nm, which is a linear function of the extent of microtubule assembly (Gaskin et al., 1974). (B) Correlation of seeded assembly with cold-stable microtubule protein concentration. Purified cold-stable and cold-labile microtubule proteins were mixed to different final ratios, but to a constant final protein concentration of 1.2 mg/mL, and challenged for assembly competency in 0.5 mM GDP, 0.05 mM GTP, and 1.0 mM AMPPNP (lines B–G). Line A, cold-stable protein only was assembled with 0.5 mM GTP as a control; line B, cold-stable protein alone; lines C–F, cold-stable protein is 0.75 (line C) of the total, 0.5 (line D), 0.33 (line E), and 0.165 (line F); line G, cold-labile protein alone. (C) Initial slopes of assembly curves in (B) have been plotted here to show that seeding is a linear function of the cold-stable microtubule protein concentration.

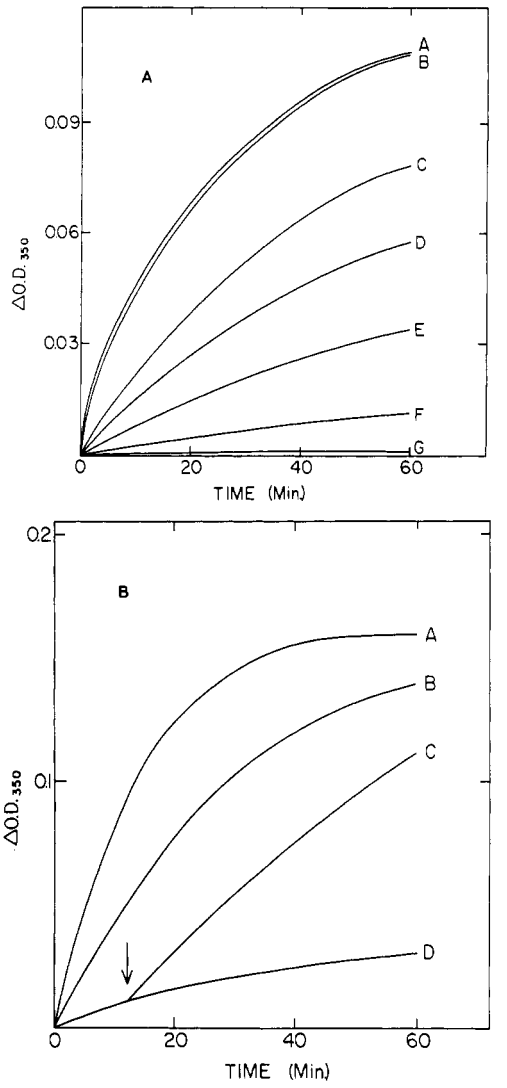

FIGURE 2: Effect of calcium pretreatment on the ability of cold-stable microtubules to seed assembly. (A) Effect of varying calcium concentrations. Cold-stable microtubule protein was treated for 50 min at 0 °C with different concentrations of free calcium and then gel filtered on G-25 columns to restore to the initial MME buffer conditions. Free calcium in MME buffer was determined as published elsewhere (Job et al., 1983). All protein concentrations were adjusted to 1.0 mg/mL prior to the assembly assay. Calcium concentrations during pretreatment are line A, none, line B, 2 μM, line C, 10 μM, line D, 0.1 mM, line E, 0.5 mM, line F, 1.0 mM, and line G, 2.0 mM. (B) Effect of shearing on seed-restricted assembly. Cold-stable microtubule protein containing seeds (second assembly cycle) was either set aside as a control or treated with 1.0 mM free calcium for 30 min at 0 °C and then desalted in MME buffer on a G-25 column. Assembly was assayed by turbidity measurement at 350 nm. Line A, control plus 0.5 mM GTP; line B, control with 0.5 mM GDP, 0.05 mM GTP, and 1.0 mM AMPPNP; line C, calcium-treated sample assembled with same conditions as for line B and then sheared at the arrow; line D, same as line C but no shearing.

Solubilization of Assembly Seeds to Seed-Forming Subunits ("Preseeds"). We have presented evidence to support the premise that superstable microtubule fragments, resistant to −80 °C, seed unfavored assembly and that these fragments are disassembled to an active subfraction in the presence of millimolar calcium or micromolar calmodulin. If these observations are correct, then a microtubule centrifugation step, applied to −80 °C treated superstable microtubule protein, should remove assembly seeds. However, preparations that are calcium treated and subsequently centrifuged should yield supernatants that restore seeding activity in the second recovery cycle.

The direct test bears out both suppositions. In the first cycle following centrifugation, the supernatant fractions of both the calcium-treated and control microtubule populations assemble

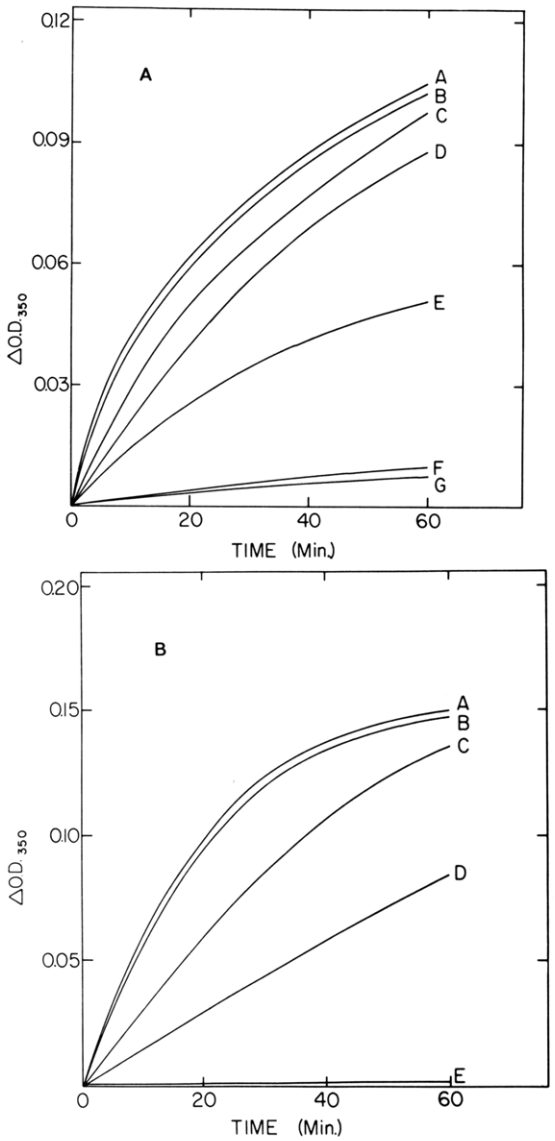

FIGURE 3: Effect of calmodulin and of salt on seeded assembly. (A) Calmodulin effect. Cold-stable microtubule protein capable of seeded assembly was incubated with 1.0 mM free calcium and increasing concentrations of calmodulin for 10 min at 0 °C, then G-25 column filtered, and assayed for competency at seeded assembly in MME buffer containing 0.5 mM GDP, 0.05 mM GTP, and 1.0 mM AMPPNP. Line A, untreated control; line B, calmodulin only (1.0 μM); line C, calcium only (1.0 mM); lines D–G, 1.0 mM free calcium and 0.1, 0.3, 0.6, and 1.0 μM calmodulin. Final protein concentration was 1.0 mg/mL. (B) Salt effect. Cold-stable microtubule protein capable of seeded assembly was incubated with various concentrations of NaCl for 1 h at 0 °C, then gel filtered into MME buffer on G-25 columns, and assayed for assembly in 0.5 mM GDP, 0.05 mM GTP, and 1.0 mM AMPPNP. Line A, untreated control; lines B, C, D, and E, 50, 100, 150, and 200 mM NaCl, respectively. The time chosen for exposure to salt is not an end point. Dissociation continues slowly for hours. Protein concentration during the assay was 1.0 mg/mL.

into partially cold stable microtubules in 1 mM GTP but cannot assemble in 10-fold excess GDP (Figure 6A). In the second cycle, only the population treated with calcium prior to centrifugation recovers its assembly seeding capability (Figure 6B). Evidently, the cold-stable microtubule fragments have disassembled in calcium to a nonsedimentable unit capable of restoring assembly seeds in a second cycle. We designate the seed-forming subunits (those active in restoring seeding activity in a second cycle of recovery) as "preseeds".

The failure of preseed activity to enter the supernatant fraction during centrifugation of seeds suggests these seed-forming subunits remain tightly associated with microtubules

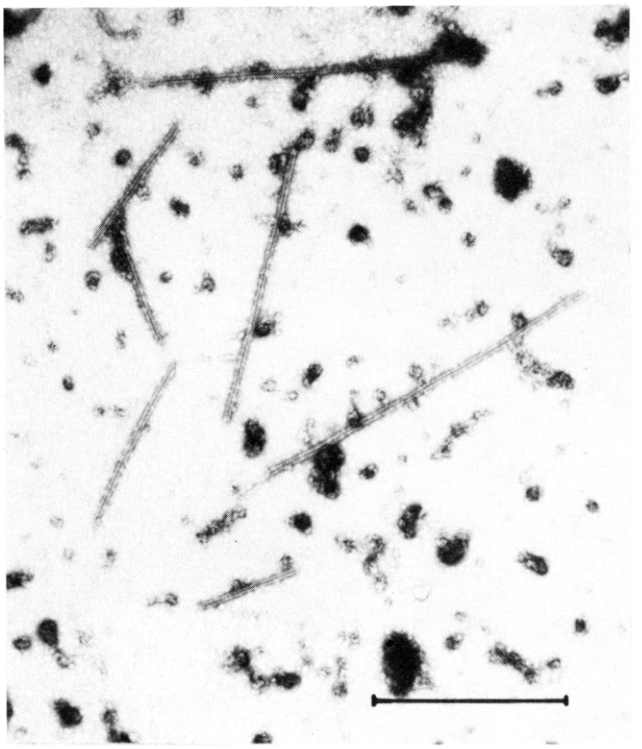

FIGURE 4: Electron microscopy image of microtubule seeds following −80 °C and sonication. Purified microtubule seeds were subjected to −80 °C and then to sonication with a machine setting of 40 W in three bursts of 15 s each at 0 °C. A protein sample was prepared for electron microscopy following sonication. Morphologically normal microtubules are abundant, even though these parameters of freezing or of sonication are each more than sufficient to effect the total disassembly of cold-stable microtubules. The bar represents 1.0 μm.

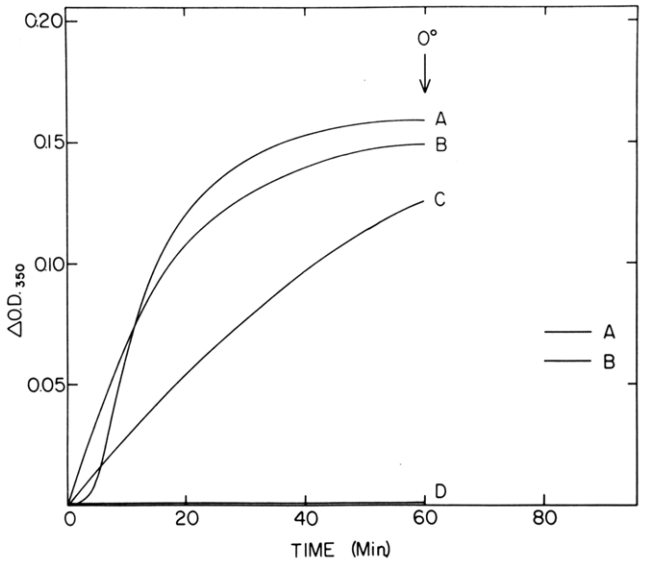

FIGURE 5: Recovery of seeds in the second cycle of assembly. Cold-stable microtubule protein, containing assembly seeds, was treated with either calmodulin or salt to disrupt seeds, then filtered on G-25 columns, assembled in GTP at 30 °C, and then gel filtered on G-25 columns again. The graph shows the second assembly cycle. Assembly in 0.5 mM GTP: line A, pretreated with 0.2 M NaCl, 2 h, 0 °C; line B, pretreated with 1.0 mM free calcium plus 5.0 μM calmodulin, 2 h, 0 °C. Assembly in 0.5 mM GDP, 0.05 mM GTP, and 1.0 mM AMPPNP: line C, high GDP assembly of the calmodulin-pretreated sample (as line B); line D, high GDP assembly of the salt-pretreated sample (as line A). The subsequent cold-stable microtubule levels for lines A and B are also shown (after 20 min at 0 °C). Protein concentration for the assay was 1.0 mg/mL.

and are not subject to rapid equilibrium loss. A direct test of this supposition was performed by diluting a superstable

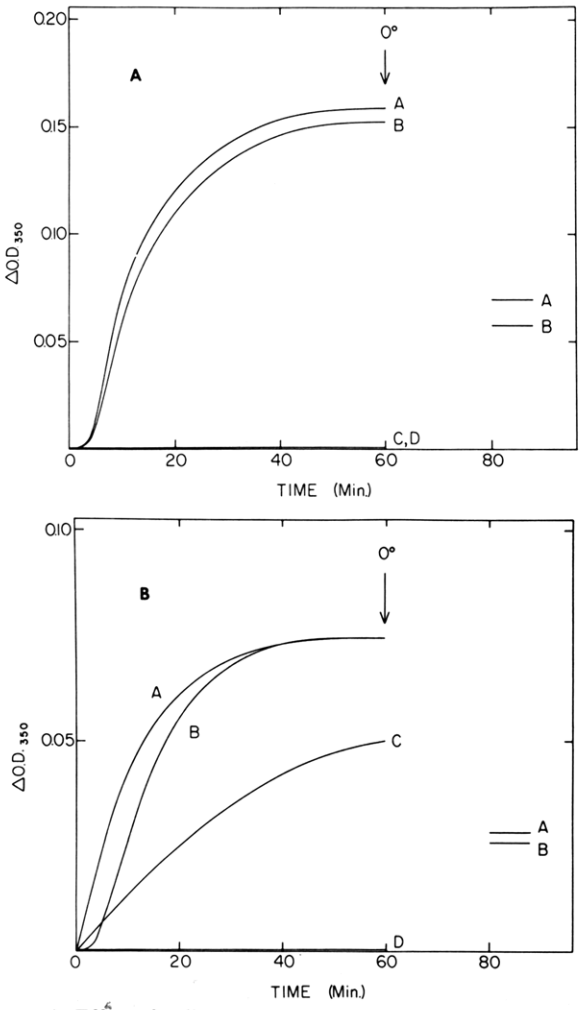

FIGURE 6: Effect of sedimentation on seeded assembly of cold-stable microtubule protein. Cold-stable microtubule protein, frozen at −80 °C and then thawed, was either set aside for a control or treated with 1.0 mM calcium and 5.0 μM calmodulin for 2 h at 0 °C. All samples were then centrifuged at 60000*g* for 30 min, and the supernatant fractions were assayed for assembly competency. (A) Assembly in the first cycle after calmodulin treatment. Line A, calmodulin-treated sample, assembled in 0.5 mM GTP; line B, nontreated control assembled in GTP; lines C and D, assembly of samples as in lines A and B, respectively, except the buffer contained 0.5 mM GDP, 0.05 mM GTP, and 1.0 mM AMPPNP. Cold-stable levels for samples A and B are shown. The cold-stable level of calmodulin-treated samples is always higher, possibly indicating the release of stabilizing blocks from superstable microtubules. (B) Assembly in the second cycle. Cold-stable microtubule protein samples were treated as indicated above, except that after the centrifugation step, they were assembled in 0.5 mM GTP, then frozen at −80 °C, then thawed at 6 °C, gel filtered on G-25 columns, and assayed. Line A, calmodulin-treated sample, assembled in 0.5 mM GTP; line B, nontreated control assembled in GTP; line C, calmodulin-treated sample assembled in 0.5 mM GDP, 0.05 mM GTP, and 1.0 mM AMPPNP; line D, nontreated control assembled in the same conditions as for line C. Cold-stable levels of samples A and B are shown. Protein concentration for the assay was 1.0 mg/mL.

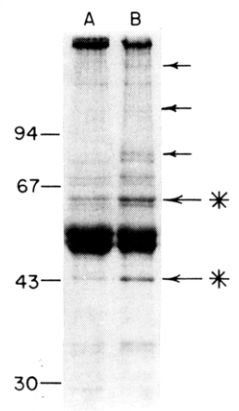

FIGURE 7: Polyacrylamide gel analysis of protein with seeding activity. Purified microtubule protein with seeding activity (lane B) is compared here with purified cold-stable microtubule protein exhibiting cold stability but no seed activity (lane A). The two protein samples were prepared identically except that the nonseeding material was derived as a supernatant following centrifugation of the seed-containing preparation, as described in the legend to Figure 6. The SDS–8% polyacrylamide gel was stained with Coomassie blue R. Arrows indicate polypeptides found only in the seeding material. Asterisks indicate abundant enhanced polypeptides found in this preseed material. Molecular weight markers are indicated at the left margin (numbers are times 10^{-3}). The purified preseed material was prepared as follows: Cold-stable microtubule protein pellets (see Materials and Methods) are diluted 1/2.5 in MME buffer, and 150 mg of protein is centrifuged at 60000*g* for 90 min. The supernatant contains nonseeding cold-stable microtubule protein. The pellet, containing concentrated seeds, is resuspended to $^1/_{20}$ the original volume in MME buffer containing 10% sucrose and centrifuged again (60000*g*) for 30 min in 2-mL tubes. The pellet of purified seeds (superstable microtubules) is resuspended in 1 mL of MME with 3 mM free calcium and 1.0 μM calmodulin. After 50 min at 0 °C, the material is centrifuged (150000*g*, 30 min). The supernatant fraction contains purified preseed activity.

microtubule fraction 10-fold for 1 h at 0 °C and comparing its assembly seeding activity with a fraction diluted into recycled cold-labile microtubule protein just prior to assay. Assembly seeding activity was indeed undiminished by prolonged exposure to dilution conditions.

DEAE ion-exchange chromatography of cold-stable microtubule protein yields a flow-through eluate fraction that contains cold-stabilizing activity. Passage of a preparation containing seed-forming subunits through a DEAE column also yields a flow-through fraction that has cold-stabilizing activity, but this fraction has no seed-forming activity. The result of such an experiment is superimposable on that already shown for loss of seed-forming activity following salt treatment of seeds (see Figure 5).

Preliminary Analysis of Seed-Forming Subunits. It is clear that microtubule assembly seeding activity is distinguishable from, but copurifies with, cold stability. The seed-forming subunits (preseeds) therefore must differ from the cold-stabilizing blocks that we have previously described. Polyacrylamide gel electrophoresis of purified microtubule preseeds was used to compare their protein composition with that of a nonseeding cold-stable microtubule preparation. There are minor polypeptides present in the preseed preparation that are absent when there is no seeding activity, and two prominent polypeptides are substantially enriched in the assembly seeding preparation (asterisks, Figure 7). However, it is impossible as yet to conclude that these differences are meaningful.

A rough estimate of the size of seed-forming subunits is of considerable interest, since it is possible that either a large integral structure enters the microtubule and stabilizes a long stretch of polymer or a cooperative assembly of superstable polymer regions from small subunits proceeds coincidently with microtubule assembly. We estimate that the seed-forming subunits must be relatively small entities, from our inability to pellet the activity upon high-speed centrifugation and from our failure to find any distinguishing structure by electron microscopy. Further, when we centrifuge preseeds into a 15–45% linear sucrose gradient, after 5 h of centrifugation at 35000*g* all of the seed-forming activity remains near the gradient interface (Figure 8) (at the position of soluble proteins). Microtubule oligomeric rings (30 S), for comparison, migrate approximately 0.33 of the total gradient distance in

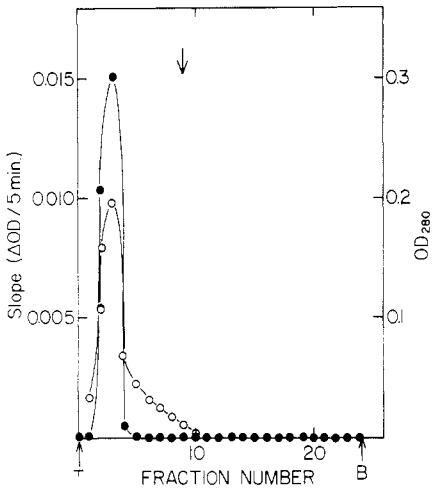

FIGURE 8: Preliminary sucrose gradient analysis of preseed size. Purified preseed activity (see the legend to Figure 7) was subjected to centrifugation on a 4.5-mL 15–45% linear sucrose gradient in MME buffer (150000*g*, 5 h, 4 °C), and the gradient fractions were assayed for preseed activity as follows: 80 μL of each 200-μL fraction was mixed with 20 μL of cold-labile microtubule protein (final concentration of 2 mg/mL), and the solution was adjusted to 5 mM EGTA. The mixture was incubated for 30 min at 30 °C in the presence of 1×10^{-4} M GTP and then immersed in liquid N_2. Cold-labile microtubule protein in MME buffer (containing GDP and AMPPNP, to yield final assay concentrations of 0.8 and 1.0 mM, respectively) and was added upon thawing to a final 300 μL of 2 mg/mL microtubule protein. The solutions were transferred to minicuvettes, and assembly was initiated by addition of GTP to a final concentration of 8×10^{-5} M, so that the final GTP/GDP ratio was 1/10. Seed activity (●) was recorded as the initial slope of assembly in the second cycle, as measured by the change in turbidity at 350 nm. Protein concentrations of gradient fractions were determined by measuring the absorbance at 280 nm of diluted aliquots (○). The arrow indicates the position to which microtubule protein rings of 30 S migrated. Soluble proteins (bovine serum albumin, glucose oxidase, and tubulin) all migrated to a peak in fraction 3, which contained the highest preseed activity. "T" and "B" represent the top and bottom of the gradient, respectively.

this time (arrow, Figure 8). The assay of seed-forming activity used is described in the legend to Figure 8.

In the recovery first assembly cycle, the seed-forming activity assembles into microtubule fragments that may act as assembly seeds in a second assembly cycle. We have assayed the time course of seed formation during this process by assembling microtubule protein, which contains seed-forming activity at 30 °C, and freezing aliquots at −80 °C to obtain time points. In a second cycle, containing 10-fold excess GDP, assembly seeding activity is assayed. We find that the rate of seed formation greatly exceeds that of assembly, but seed formation begins about 1 min after microtubule assembly initiates (Figure 9A). Seed formation also occurs rapidly upon preseed addition to steady-state microtubules (Figure 9B). It is apparent that assembly seeds are forming by addition of constituents on preformed microtubules even in the presence of GDP 10-fold in excess of GTP (the condition preventing new assembly starts).

Discussion

It is now apparent that two subclasses of cold-stable microtubules may be isolated from mammalian brain tissue. Both classes share some properties, but they are clearly distinguishable on the basis of others.

The superstable microtubule assembly seeds represent a remarkable subpopulation. Micrometer lengths of these microtubules strongly resist −80 °C and sonication (Figure 4) although micromolar calmodulin is sufficient to cause their

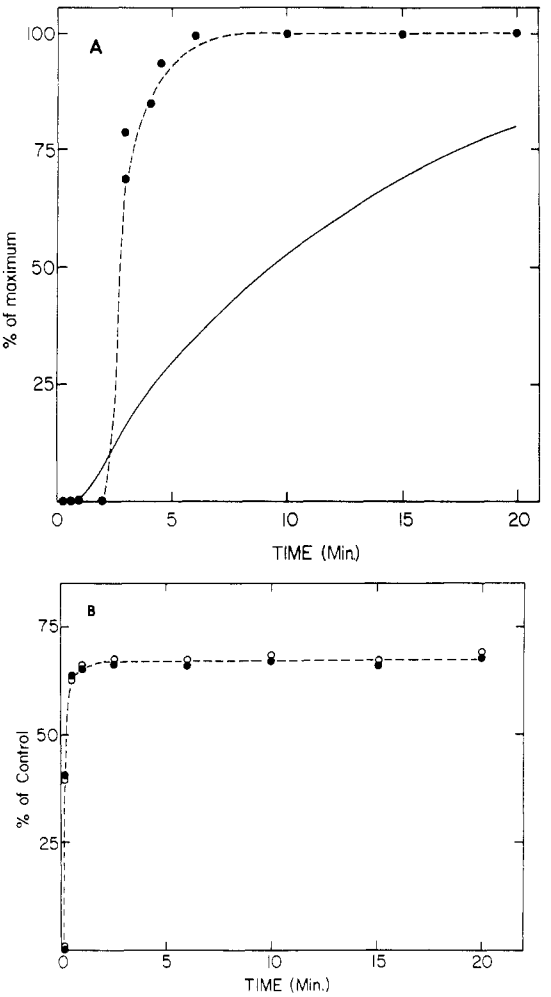

FIGURE 9: Kinetics of seed formation from preseed activity during microtubule assembly. (A) Assay of seed formation under assembly initiating conditions. Microtubule protein with preseed activity was obtained by treating seed containing cold-stable microtubules with 2.0 mM calcium, as described in Figure 2. The protein was assembled at 5 mg/mL after addition of 5 mM EGTA and 1×10^{-4} M GTP. The assembly kinetics, as assayed by turbidity, are shown (solid line). Aliquots were removed at the indicated times, immersed in liquid N_2, thawed at 6 °C, passed through G-25 columns, and subsequently assayed for seed activity at 2 mg/mL as described in the legend to Figure 2. The initial slopes of assembly in GDP/GTP ratios of 10/1 are plotted (as a percent of the maximum slope obtained) (●). (B) Assay of seed formation on preassembled microtubules. Preseeds were purified as described in the legend to Figure 7 and assayed as indicated in the legend to Figure 8, with modifications as described below. To assay the formation of seeds with steady-state microtubules in GTP, cold-labile microtubule protein was assembled alone for 30 min, and then a preincubated preseed preparation was added. Time points were taken by immersion of aliquots in liquid N_2 and assayed for seeds in a second assembly cycle containing 10-fold excess GDP. To assay for the formation of seeds with steady-state microtubules in 10-fold excess GDP, cold-labile microtubule protein was assembled to steady state, then GDP and AMPPNP were added, and finally preincubated preseeds were added. Time points were taken by freezing aliquots in liquid N_2 and assaying for seeds in a second assembly cycle. The data plotted represent initial slopes of assembly in the seed assay, as described under Figure 8, and are expressed as the percent of maximal preseed activity of a control assembled with preseeds continuously present (all other conditions being held constant). (●) Preseeds added in the presence of GTP only; (○) preseeds added in the presence of 10-fold excess GDP.

rapid dissociation. They then re-form rapidly from preseeds in what appears to be a cooperative association reaction. We have taken advantage of their rapid and reversible dissociation in micromolar calmodulin or millimolar calcium at 0 °C to develop a preseed purification scheme involving microtubule

assembly cycles (Figure 7, figure legend).

It is not possible as yet to distinguish if superstable microtubules are a subset of cold-stable microtubules or if the two phenomena are unrelated. If microtubule assembly seeds are a subset of cold-stable microtubules, the difference between the two possibly may be ascribed to additional polypeptides associated with the stabilizing proteins. Some differences in protein composition are observed on gels, but we do not as yet know if these differences are meaningful. The permanent loss of activity following moderate salt exposure suggests a loss of higher order structure in interacting polypeptides, since all the original macromolecular components are present after this treatment. A role for a small cofactor also cannot be ruled out, since all salt treatments involve a gel filtration step for desalting prior to reassembly and this step would remove any small dissociated ligands.

The stability of a region of the polymer (creating microtubule fragments that may act as seeds) must be due to relatively long range cooperative interactions between polypeptides. Such interactions do not exist in cold-stable microtubules lacking a seed function. We believe, instead, that cold stability is due to relatively rare blocks that are randomly dispersed on the polymer and serve to protect polymer regions between two blocks against endwise disassembly (Job et al., 1982).

The mechanism by which such stable regions are generated during preseed to seed transition is beginning to emerge. It is apparent that preseeds are not likely to be substantially larger than the size of individual polypeptides on the basis of sucrose gradient analysis. Their interaction during microtubule assembly to form stable seeds is rapid, exceeding substantially the overall pace of assembly. These interactions appear to occur with equal facility during assembly and at microtubule steady state. The reaction will occur at steady state when GDP exceeds GTP by 10-fold (the condition in which new microtubule starts will not form but preexisting microtubules will elongate) but will not occur under these conditions in an unassembled microtubule protein solution. Seed formation therefore appears to require a preexisting microtubule template.

The seeds that we have isolated could obviously have a role in seeding microtubule assembly in the intracellular environment. Microtubules initiate assembly usually only at restricted sites such as cell centers (Pickett-Heaps, 1975; Snyder & McIntosh, 1976). One interpretation of the intrinsic treadmilling property of microtubules is that the resulting disequilibria at the two polymer ends restrict net microtubule assembly only to sites where the net disassembly end is stabilized against subunit loss (Kirschner, 1980). The superstable seeds that we have isolated exhibit both functions; they form polymer regions that are extremely stable to disassembly loss, and they seed microtubule assembly under unfavorable conditions.

The rapid coalescence of seeds from preseeds in an apparent cooperative manner suggests that they may form rapidly at proper sites in the cell. If we are, indeed, working with a genuine seeding reaction, the fact that seed formation and dissolution are regulated by calmodulin and by ATP (unpublished observation) suggests the manner by which the cell might temporally regulate assembly and disassembly and, additionally, cause stable seeded microtubules to become rapidly treadmilling structures by removal of their end blockage.

Acknowledgments

We acknowledge the fine technical assistance of Françoise Gilles and the assistance of Charles T. Rauch in preparation of the figures.

Registry No. Calcium, 7440-70-2.

References

Asnes, C. F., & Wilson, L. (1979) *Anal. Biochem. 98*, 64–73.

Dustin, P. (1978) *Microtubules*, Springer, New York.

Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–758.

Grisham, L. M. (1976) Dissertation, Stanford University, Stanford, CA.

Job, D., Fischer, E. H., & Margolis, R. L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4679–4682.

Job, D., Rauch, C. T., Fischer, E. H., & Margolis, R. L. (1982) *Biochemistry 21*, 509–515.

Job, D., Rauch, C. T., Fischer, E. H., & Margolis, R. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3894–3898.

Kirschner, M. W. (1980) *J. Cell Biol. 86*, 330–334.

Margolis, R. L., & Wilson, L. (1978) *Cell (Cambridge, Mass.) 13*, 1–8.

Margolis, R. L., & Rauch, C. T. (1981) *Biochemistry 20*, 4451–4458.

Margolis, R. L., & Wilson, L. (1981) *Nature (London) 292*, 705–711.

Pickett-Heaps, J. D. (1975) *Ann. N.Y. Acad. Sci. 253*, 352–372.

Pirollet, F., Job, D., Fischer, E. H., & Margolis, R. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1560–1564.

Roberts, K., & Hyams, J. S., Eds. (1979) *Microtubules*, Academic Press, London.

Snyder, J. A., & McIntosh, J. R. (1976) *Annu. Rev. Biochem. 45*, 699–720.

Timasheff, S. N., & Grisham, L. (1980) *Annu. Rev. Biochem. 49*, 565–592.

Webb, B. C., & Wilson, L. (1980) *Biochemistry 19*, 1993–2001.