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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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sition of highly purified cold-stable and cold-labile material. The two preparations differ by the presence of a few unique proteins seen only in the cold-stable subpopulation. Furthermore, dissociated cold-stable microtubule protein reassembles entirely into cold-labile material after passage through a column of immobilized calmodulin in which these unique proteins are retained. A model is presented for the generation of cold-stable material upon recycling, based on the random distribution of these blocks in newly polymerized microtubules.

Experimental Procedures

Materials. Material for buffers and other reagents were obtained from Sigma Chemicals, Inc. and, unless stated otherwise, were of the purest grade available.

Calmodulin was purified from beef and pork brain by modifications of methods previously published (Watterson et al., 1976). Podophyllotoxin (PLN)[1] was obtained from Aldrich. The buffer used (designated as MEM) was composed of 100 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), 1.0 mM EGTA, and 1.0 mM $MgSO_4$, pH 6.75.

The calmodulin affinity column was prepared by cross-linking beef brain calmodulin to Affi-Gel 15 (Bio-Rad) according to Erlich et al. (1979) and Alwine et al. (1979). The final material contained 2 mg of immobilized calmodulin/mL of gel.

Assembly–Disassembly Assays by Turbidimetric Measurements. Assembly assays were performed in 0.8-mL cuvettes. Changes in the optical density of microtubule solutions were followed at 350 nm, 30 °C, by using a Varian Cary 219 recording spectrophotometer equipped with a constant temperature chamber and a cell programmer for rapid successive assays of five samples. Assembly was initiated by addition of GTP (to 0.1 mM final concentration) to samples previously adjusted for the base line in the spectrophotometer. Cold-stable material was routinely examined by electron microscopy to confirm that it indeed consisted of microtubules.

Purification of Cold-Stable Microtubules. Cold-stable microtubules were purified from the brain of adult rats (inbred strains W/FU or Sprague Dawley) according to previously published procedures (Margolis & Rauch, 1981; Job et al., 1981). The purified cold-stable microtubules either were used directly for disassembly assays or were depolymerized by methods described below. The disassembled cold-stable microtubule protein was centrifuged (40000*g* for 30 min at 4 °C), and the supernatant was adjusted to 0.1 mM GTP and to a concentration of EGTA at least 2-fold in excess of that of free calcium prior to the assembly assay.

Other Methods. Electron microscopy was performed as previously reported (Margolis & Wilson, 1978). Slab gel electrophoresis was performed by using 8% polyacrylamide and 0.1% sodium lauryl sulfate, according to Schier-Neiss et al. (1978). Gels were stained with Coomassie blue R.

Results

Cold Disassembly of Sheared Cold-Stable Microtubules. Cold-labile microtubules, selected by cycles of temperature-dependent assembly–disassembly, are in a state of equilibrium with their constituent subunits at physiological temperature. They rapidly ($t_{1/2}$ = 2 min) disassemble at 0 °C. By constrast, the cold-stable subpopulation does not release its subunits either at warm or at cold temperatures (Webb & Wilson, 1980; Margolis & Rauch, 1981; Job et al., 1981). Two possible mechanisms might account for this failure to exchange subunits: it might be due either to an altered state of the subunits in the cold-stable polymer or to certain rare blocks that protect the otherwise cold-labile polymer from end-wise depolymerization.

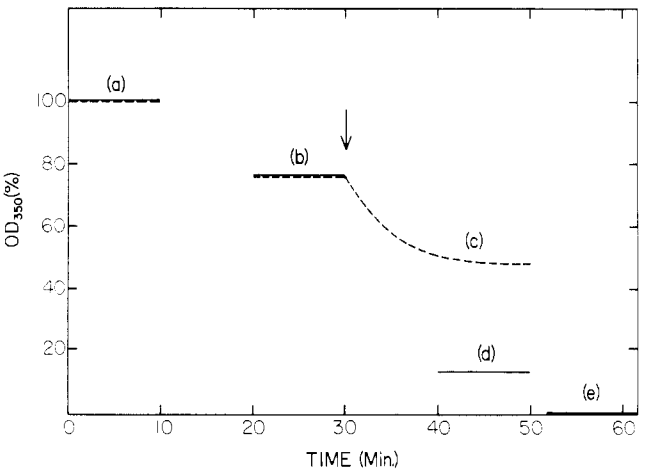

FIGURE 1: Effect of shearing on cold-stable microtubules. Sucrose cushion purified cold-stable microtubule solutions were placed in cuvettes, previously adjusted to base line against buffer, and incubated at 30 °C. Optical density of unsheared microtubules is taken as 100% (a). Microtubules (average length 5.21 ± 0.50 μm) were then sheared to 1.10 ± 0.16 μm average length by three passages through a 1-mL syringe with a 26-gauge needle (b). After 10 min, microtubules were either exposed to 50 μM PLN (added at arrow) at 30 °C (c) or chilled to 0 °C for 10 min and rewarmed in the presence of 50 μM PLN (d). The base line was established by totally depolymerizing microtubules in 20 mM $MgCl_2$ (e). Relative optical densities were 76% for (b), 45% at plateau for (c), and 15% for (d).

Purified cold-stable microtubules were subjected to mild shearing in order to reduce their average lengths approximately 5-fold to test which of these mechanisms is correct. If the entire polymer were cold stable, introduction of new ends should not affect polymer stability. If, however, end caps did protect an otherwise cold-labile polymer from disassembly, shearing should nick the polymer between the blocks and result in a rapid cold-induced depolymerization.

Purified cold-stable microtubules that will not disassemble in the cold or in the presence of an assembly-inhibiting drug such as podophyllotoxin (PLN) (Figure 1, line a) will rapidly depolymerize after shearing when exposed to either PLN (line c) or cold temperature (line d). Therefore, shearing is an effective means of disassembling cold-stable microtubules. The results illustrated in Figure 1 were confirmed independently both by analysis of centrifuged microtubule pellets representing each step of the procedure and by the filter assay of microtubules fully labeled with [^{3}H]GTP (Wilson et al., 1981). These procedures, together with electron microscopy, all confirm that no microtubules or protein aggregates are present in the totally depolymerized material (base line, line e) following 20 mM $MgCl_2$ treatment. They further establish that a drop in microtubule quantity occurs with shearing (line b) which could be attributed to a transition of nonequilibrium cold-stable microtubules to an equilibrium state upon shearing.

Characteristics of Reassembled Cold-Stable Microtubules. Centrifugation (at 4 °C) of the sheared, cold-depolymerized product yields disassembled microtubule protein in the supernatant that can rapidly reassemble upon addition of 0.1 mM GTP at 30 °C (Figure 2, a). The microtubules that are generated represent a mixed population of both cold-stable and cold-labile material. Chilling to 0 °C for 10 min reveals a 42%

[1] Abbreviations: Ca^{2+}-calmodulin, the active form of calmodulin with four occupied Ca^{2+} binding sites; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MEM buffer, 100 mM Mes [2-(*N*-morpholino)ethanesulfonic acid], 1.0 mM EGTA, and 1.0 mM $MgSO_4$, pH 6.75; PLN, podophyllotoxin; STOPs, stable tubule only polypeptides (polypeptides associated only with cold-stable microtubules).

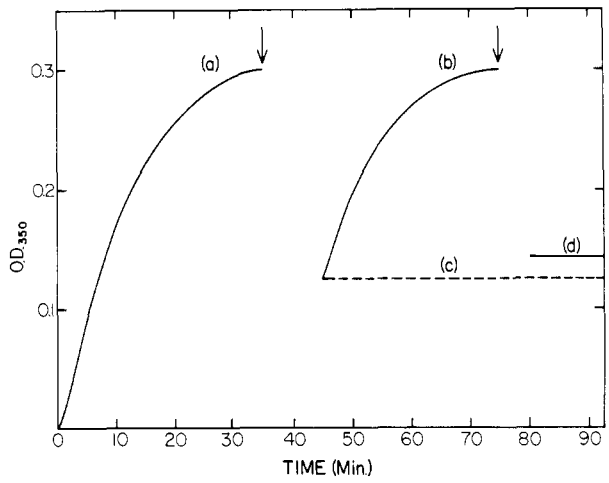

FIGURE 2: Recycling of cold-stable microtubule protein after dissociation by shearing. Sucrose cushion purified cold-stable microtubules were sheared by three passages through a 1-mL syringe fitted with a 26-gauge needle and depolymerized by chilling to 0 °C. Microtubule protein was then isolated as a supernatant following centrifugation (40000*g*, 30 min, 4 °C). Reassembly of the supernatant was monitored after warming to 30 °C in the presence of 0.1 mM GTP (a). At the first arrow, the microtubule solution was chilled to 0 °C for 10 min. One aliquot (b) was then rewarmed for a second assembly cycle; the other (c) was rewarmed in the presence of 50 μM PLN to determine the first cycle cold-stable microtubule population. Sample b was chilled to 0 °C at the second arrrow and rewarmed in the presence of 50 μM PLN to determine the second cycle cold-stable level (d). Following the experiment, the base line was rechecked by addition of 20 mM $MgCl_2$, which causes total disassembly of all microtubules (not shown). Cold-stable material following one cycle (c) was 42% of sample a and was 47% of sample b after two cycles (d).

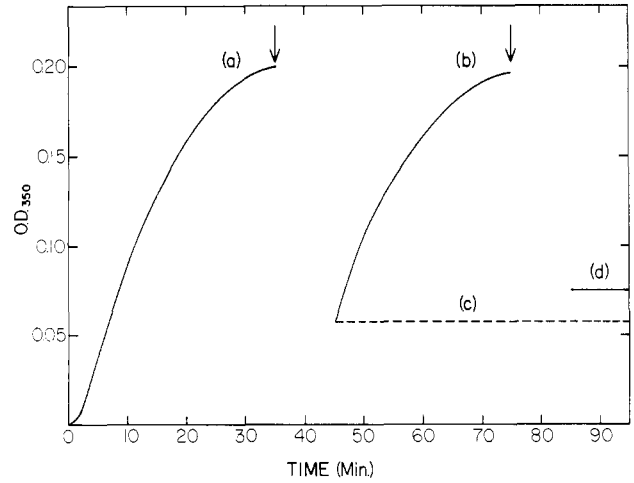

FIGURE 3: Recycling of cold-stable microtubule protein after dissociation by Ca^{2+}-calmodulin. Sucrose cushion purified cold-stable microtubules were brought to 1.5 mM $CaCl_2$ (0.5 mM free Ca^{2+}) in the presence of 5.0 μM calmodulin. After 10 min at 0 °C, the solution was centrifuged at 40000*g* for 30 min at 4 °C. To the supernatant were added 2 mM EGTA and 0.1 mM GTP. Assembly of two samples at 30 °C was then monitored as described in Figure 2. (a) First assembly cycle. The samples were chilled to 0 °C for 10 min (first arrow), and one (b) was warmed to 30 °C for reassembly. The other sample (c) was warmed to 30 °C in the presence of 50 μM PLN. At the second arrow (b), the reassembled sample was chilled again to 0 °C for 10 min. (c) represents sample b after rewarming in the presence of 50 μM PLN. The first cycle cold-stable level (c) is 29% of that of (a); the second cycle cold-stable level (d) is 37% of that of (b).

subpopulation that remains cold stable and drug resistant. Reassembly again occurs (Figure 2, b) upon rewarming of the cold-disassembled material. When chilled once more (Figure 2, d), the cold-stable population increases slightly to 47% of the total. This result is characteristic of many experimental repeats: the microtubules that re-form are never entirely cold stable nor entirely cold labile. If, however, the cold-stable microtubules are removed from the solution by centrifugation at this stage, the remaining supernatant reassembles into entirely cold-labile material.

Reassembly Characteristics of Calmodulin-Disassembled Cold-Stable Microtubules. It was previously demonstrated that cold-stable microtubules disassemble rapidly in the presence of low levels of Ca^{2+}-calmodulin (Job et al., 1981), suggesting that the cold-stabilizing factor(s) might interact with calmodulin. Two questions may be asked, i.e., whether (a) the disruption of cold stability by calmodulin is reversible and whether (b) reassembly of the material generated under these conditions might yield only partial cold stability in the next cycle.

To test these possibilities, we added 5 μM calmodulin and 0.5 mM free calcium to purified cold-stable microtubules, and the resulting depolymerized material was separated by centrifugation at 4 °C. Upon addition of excess EGTA and incubation of the supernatant at 30 °C (Figure 3), the repolymerized microtubules behaved nearly identically with the material obtained from sheared cold-stable microtubules as depicted in Figure 2. That is, the reassembled product was only partially (29%) cold stable; a second assembly cycle (Figure 3, b) somewhat increased the yield in cold-stable material to 37% (Figure 3, d).

It is clear from these results that purified cold-stable microtubules can be rendered cold labile by at least two procedures, namely, shearing and calmodulin treatment. In both instances, the disassembled microtubules can be reassembled

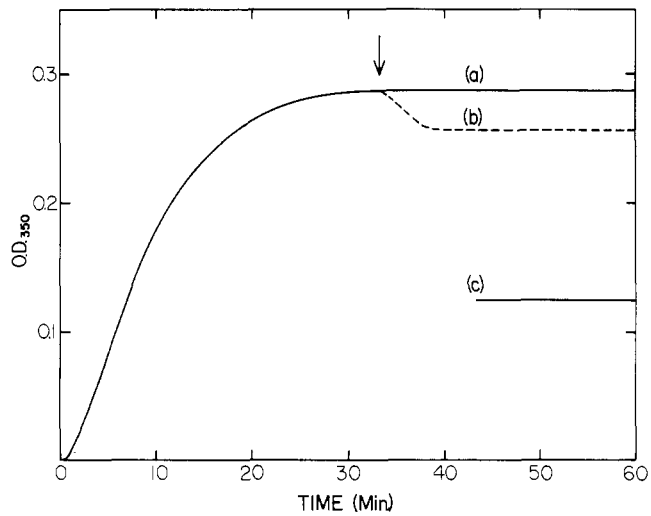

FIGURE 4: Relative cold and drug sensitivity of microtubules recycled from a cold-stable population by shearing. Sucrose cushion purified cold-stable microtubules were sheared, chilled, and centrifuged as described for Figure 2. The supernatant was brought to 0.1 mM GTP and reassembled by warming to 30 °C. (a) Reassembled and untreated microtubule control. (b) Sample exposed to 50 μM PLN at arrow (30 °C). (c) Sample chilled to 0 °C for 10 min at arrow and then rewarmed in the presence of 50 μM PLN. Drug-stable microtubules (b) are 90% of control (a), and cold-stable microtubules (c) are 43% of control (a).

into partially cold-stable material. In these experiments, the extent of drug (PLN) insensitivity was always considerably greater than the degree of cold stability, as shown in Figure 4 for cold-stable microtubules recycled once after shearing. Here, the cold-stable subpopulation had become 43% cold resistant and 90% drug resistant.

Comparative Composition of Cold-Stable and Cold-Labile Microtubules. This was carried out by gel electrophoresis on material carefully purified by two assembly–disassembly cy-

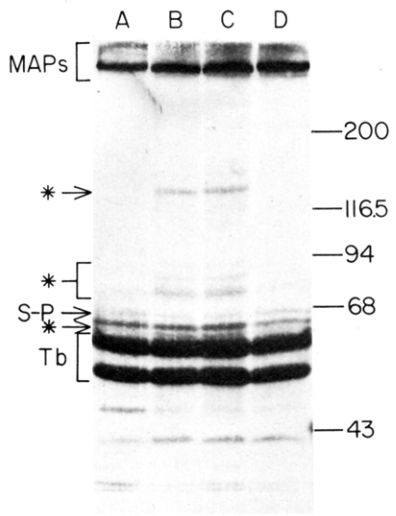

FIGURE 5: Dodecyl sulfate–polyacrylamide gel electrophoresis of microtubule protein before and after passage through a calmodulin column. (A) Cold-labile material purified as described below. (B) Purified cold-stable material prior to calmodulin column. (C) "Cold-stable" eluate from calmodulin affinity column run in the presence of excess EGTA. (D) "Cold-labile" eluate from calmodulin affinity column run in the presence of excess $CaCl_2$. Asterisks, the 135-, 70–82- and 56-kilodalton STOP polypeptides; Tb, α- and β-tubulins; MAPs, microtubule-associated proteins; S-P, switch protein. Molecular weight standards are indicated on the right. Each lane contained 100 μg of protein. Cold-labile microtubules (lane A) were purified by two cycles of assembly. In the first cycle, at the point of maximal cold lability (20 min), all assembled microtubules were collected by centrifugation through a 50% sucrose cushion. The pellet was gently resuspended in MEM buffer at 0 °C for 10 min to avoid any shearing, and the resulting suspension was centrifuged at 40000*g* for 30 min at 4 °C. The supernatant was brought to 0.1 mM GTP, assembled at 30 °C for 30 min, and recentrifuged through a 50% sucrose cushion. The resulting pellet was again gently resuspended at 0 °C and centrifuged as above. The supernatant contains 100% cold-labile microtubule protein. Cold-stable microtubules (lane B) were purified by two cycles of assembly with an intervening shearing step as described for Figure 2. The second cycle cold-stable microtubules were finally isolated by chilling to 0 °C for 10 min, rewarming in 50 μM PLN, and centrifuging through a 50% sucrose cushion.

cles. The presence of a 64-kilodalton polypeptide (switch protein) that is uniquely dephosphorylated in the cold-stable subfraction of the rat brain crude extract was previously reported (Margolis & Rauch, 1981). Results are shown in lanes A and B, respectively, of Figure 5. The switch protein (labeled S-P in Figure 5) is present in equal quantity in each of the two recycled preparations, though presumably phosphorylated in one sample and dephosphorylated in the other. On the other hand, there is a clear and reproducible difference in the presence of a number of polypeptides seen only in recycled cold-stable fractions (Figure 5, lane B, top asterisk), including one of approximately 135 kilodaltons, a group of three polypeptides in the 70–82-kilodalton range, and a polypeptide of 56 kilodaltons (lowest asterisk in Figure 5; the band can be seen just above α-tubulin). We refer to these proteins collectively as STOPs (stable tubule only polypeptides) though at this point we do not know which, if any, of these is involved in the process of cold stabilization.

Transformation of Cold-Stable Microtubule Proteins to Cold-Labile by Passage through a Calmodulin Affinity Column. Since calmodulin was shown to cause the rapid disassembly of cold-stable microtubules (Job et al., 1981), one could assume that passage of cold-stable microtubules through a Ca^{2+}-calmodulin column would result in the binding of the cold-stabilizing factor(s) and render the eluate cold labile. This result was precisely obtained.

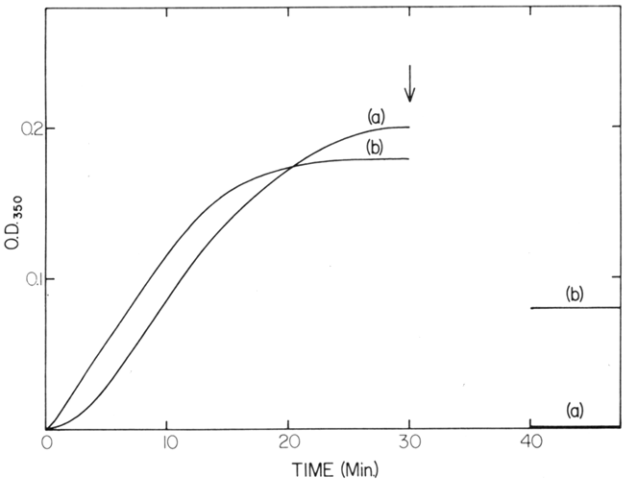

FIGURE 6: Conversion of cold-stable to cold-labile microtubule protein by passage through a Ca^{2+}-calmodulin affinity column. Cold-stable microtubules were purified as detailed in Figure 2 and then sheared by repeated passage through a 26-guage needle at 0 °C to bring about depolymerization. After centrifugation (40000*g*, 30 min at 4 °C), 0.1 mM phenylmethanesulfonyl fluoride was added, and the supernatant was divided in two. One fraction was passed through a 2-mL calmodulin affinity column in MEM buffer in the presence of 0.5 mM excess $CaCl_2$ (a) and the other in MEM buffer alone (b). Both eluates were brought to identical buffer conditions by addition of Ca^{2+} or EGTA, equilibrated to 30 °C in the spectrophotometer, and then assembled with 0.1 mM GTP. At the arrow, both samples were chilled for 10 min at 0 °C in the presence of PLN. Sample a (that had gone through the Ca^{2+}-calmodulin column) is entirely cold labile; sample b is 40% cold stable.

Purified cold-stable microtubules were sheared, cold disassembled, and centrifuged. The supernatant material was divided in two; one-half was run through a calmodulin affinity column in MEM buffer in the absence of Ca^{2+} ions, the other in the presence of 0.5 mM free calcium. The unbound material emerging in the breakthrough fractions was brought to identical buffer conditions by addition of either 0.5 mM Ca^{2+} or 1 mM EGTA. All operations were performed at 0–4 °C. The resulting solutions were then assembled at 30 °C.

The assembly curves of the two eluates are characteristically and reproducibly different (Figure 6). The eluate obtained in the absence of Ca^{2+} assembles with almost no lag, whereas the one collected in the presence of Ca^{2+} always shows a lag before assembly initiates. Likewise, upon chilling of these samples and rewarming in the presence of PLN, one finds that the first eluate has remained 40% cold stable, while the second has become entirely cold labile. Electron microscopy confirms that the cold-stable material consists of microtubules.

Analysis of the column eluates by gel electrophoresis (Figure 5, lanes C and D) reveals that the same groups of STOP polypeptides which comigrate with the purified cold-stable microtubule have been retained by the Ca^{2+}-calmodulin affinity column, further emphasizing the correlation that must exist between some of these polypeptides and cold stability.

Discussion

Working Hypothesis on the Mechanism of Microtubule Cold Stabilization. All evidence indicates that microtubule cold stabilization is due to substoichiometric blocks that are dispersed along the length of the microtubule. Shearing of cold-stable microtubules to one-fifth of their original length causes them to become 85% cold labile. The cold-stabilizing factor has not been inactivated by this procedure, since repolymerization of the sheared material reestablishes a cold-stable subpopulation. In a similar fashion, exposure of cold-stable microtubules to substoichiometric amounts of Ca^{2+}-

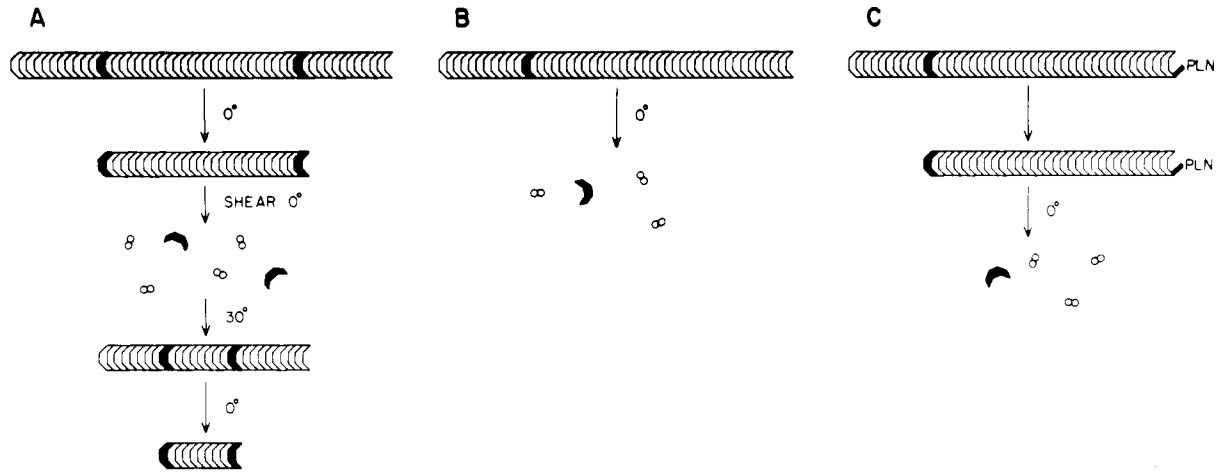

FIGURE 7: Model of microtubule cold stabilization by substoichiometric blocks. (A) When two or more blocks are present within a polymer, disassembly at 0 °C proceeds from both ends to the blocks. Shearing introduces breaks in the polymer between the blocks and leads to further depolymerization at 0 °C. Reassembly at 30 °C causes a random reshuffling of blocks, leading to only fractional cold stability in the next cycle. (B) When only one block exists within the polymer, it is totally depolymerized by cold temperature. Random reshuffling of blocks from this population in the next cycle can reestablish a multiple blocked subpopulation. (C) When only one block exists within the polymer and it is exposed to podophyllotoxin, disassembly will proceed from the unblocked end only up to the cold-stable block; since PLN "caps" the other end of the polymer at 30 °C, this singly blocked material will be stable to the drug. Cold temperature will release the PLN block and cause total disassembly of this subpopulation. Truncated chevrons schematically represent the microtubule as a polar polymer (the chevrons pointing toward the net steady-state disassembly end); filled chevrons represent cold-stable blocks; PLN represents the net assembly end blocked by podophyllotoxin; double circles represent the dimeric protein subunits of the polymer.

calmodulin leads to their rapid disassembly; reassembly of the isolated subunits in the presence of excess EGTA restores a cold-stable subpopulation.

The mechanism of cold stabilization can be pictured as follows. Rare blocking complexes are incorporated randomly into the polymer during microtubule assembly. Since disassembly induced by cold or Ca^{2+} proceeds from both ends inward (Karr et al., 1980a,b), any region of a microtubule lying between two such blocks will be protected (Figure 7A). When, however, the polymer is clipped between two blocks (as by shearing), new unblocked ends are created, and disassembly can proceed until only depolymerized subunits and blocks remain. Upon reassembly, the blocks will be randomly reintroduced into the polymer so that, again, only those portions of a microtubule that lie by chance between two blocks will become cold stable (Figure 7A).

The population of microtubules which contains only one block will undergo total disassembly in the cold or in the presence of Ca^{2+} (Figure 7B). Such a subpopulation of cold-labile microtubules can yield some cold-stable material upon reassembly, since random redistribution of blocks will produce some microtubules with two blocks or more.

PLN forms a cap at the assembly end of a microtubule at 30 °C in the presence of GTP (Margolis, 1981), slowing greatly the rates of dimer association–dissociation at this site. Meanwhile, disassembly continues unhindered at the opposite end (Margolis & Wilson, 1978, 1979; Margolis, 1981). Thus, a single block will hinder the PLN-induced disassembly of a microtubule. PLN will serve to create a second (assembly end) block at 30 °C (Figure 7C). Since the PLN block is cold labile, a portion of the mixed cold-stable/cold-labile microtubule population will be drug stable but cold labile. This prediction is in accord with the present data which consistently show more drug than cold stability in a mixed microtubule population.

Consequences of a Poisson Distribution of Cold-Stabilizing Blocks. With the assumption that the distribution of cold-stabilizing blocks on a microtubule is random, probability theory predicts that such blocks will be distributed with equal spacing along a microtubule. On the average, one block will lie at the halfway point of a polymer, two blocks at one-third intervals, three at quarter intervals, etc. As a consequence, a polymer with two blocks will be protected from cold disassembly over one-third its length; one with three blocks, over half its length, etc. Since the sampling population is, for all practical purposes, infinite, the influence of variance upon the mean in the distribution of blocks on a microtubule becomes negligible. With these considerations, the probability of cold stability, $P(S)$, in a mixed microtubule population becomes

$$P(S) = \sum_{n=1}^{n=n} \frac{n-1}{n+1} P(n) \quad (1)$$

where $P(n)$ is the probability of n blocks occurring per polymer, with $n \geq 1$. In expanded form, this becomes

$$P(S) = 0.33P(2) + 0.50P(3) + 0.60P(4) + \ldots + \frac{n-1}{n+1}P(n) \quad (2)$$

Simiarly, the total probability of cold lability, $P(L)$, is

$$P(L) = 1 - P(S) \quad (3)$$

For randomly distributed blocks, $P(n)$ is determined by the Poisson distribution function:

$$P(n) = \frac{\mu^n}{n!} e^{-\mu} \quad (4)$$

where μ is the mean number of blocks per microtubule in the population.

Thus, the probability of cold stability becomes

$$P(S) = 0.33\frac{\mu^2}{2}e^{-\mu} + 0.50\frac{\mu^3}{6}e^{-\mu} + \ldots + \frac{n-1}{n+1}\frac{\mu^n}{n!}e^{-\mu} \quad (5)$$

A similar calculation can be made for $P(L)$. Since the Poisson distribution contains only one unknown (μ), this parameter can be determined once one knows either $P(L)$ or $P(S)$.

Knowing μ, one can predict the extent of drug stability in the population if for each polymer, in addition to the cold-stable region, the distance between the net assembly end and

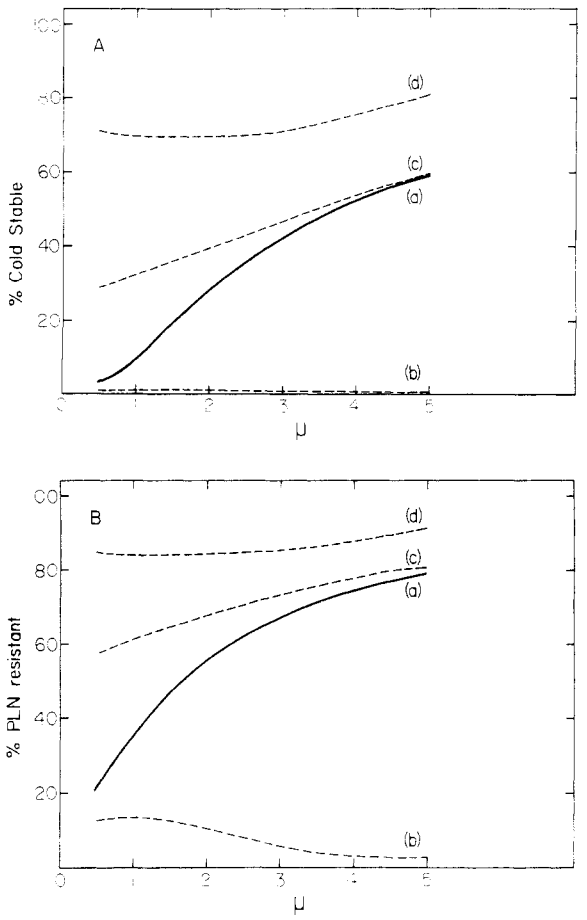

FIGURE 8: Prediction of microtubule cold stability during crude extract assembly based on the random distribution of blocks. μ represents the mean number of blocks per microtubule *in the first cycle* microtubule population. (Panel A, a) Percent cold stability in the first cycle of microtubule assembly, predicted on the basis of the mechanism proposed for cold stability and Poisson calculations described in the text. (b) Cold stability in the second cycle if only the cold-labile microtubule protein was recycled as described in the legend of Figure 5. (c) Cold stability in the second cycle, when the cold-stable material following the first cycle assembly is recycled by shearing, assuming that the microtubule *number* remains constant between the first and second cycles (i.e., that blocks participate in seeding microtubule assembly in the second cycle). (d) Same as for (c), but with the assumption that microtubule *length* remains constant between the first and second cycles (i.e., that blocks do not partipicate in seeding microtubule assembly in the second cycle). (Panel B) A similar prediction was made for the degree of podophyllotoxin sensitivity of recycling microtubule populations according to assumptions stated in the text. The lines represent the same microtubule populations as in (A).

the nearest block is protected.

Based on the above assumptions, one can generate hypothetical cold-stability and drug-stability predictions as a function of μ in the first assembly cycle (Figure 8). Some of these have already been borne out by work presented herein and unpublished observations. For instance, for μ values of $1 \leq \mu \leq 5$, a range one usually finds in the rat brain crude extract, one finds that (a) cold stability does not involve more than 50% of the population, (b) for $\mu > 3$, cold stability does not increase greatly on recycling the cold-stable subpopulation, (c) cold stability in the next cycle is negligible for all μ values if one recycles only the cold-labile subpopulation, (d) drug stability is always considerably greater than cold stability for all μ values (see Figures 1 and 4), and (e) drug stability can be substantial even when cold stability is negligible (for example, compare lines a and b at low μ in Figure 8, parts A and B).

One can further predict the average reduction in length of microtubules upon chilling for an observed percentage of cold stability. A skew of results from prediction could occur if the blocks were to participate in seeding assembly, a possibility suggested by the result in Figure 6 which shows cold-stable microtubule assembly initiating much more rapidly than cold-labile assembly. If microtubule assembly were to initiate independently of blocks, then microtubules should be of constant length, plus or minus blocks, but if blocks were to seed assembly, then the number of cold-stable microtubules would be a direct function of block concentration, and their average length would vary. We have calculated predictions for these two extremes (Figure 8A,B, lines c and d). Preliminary results fall between these values, indicating that we are dealing with a mixed population of block-seeded and unseeded cold-stable microtubules.

Since shearing leads to a redistribution of cold-stabilizing blocks and the procedure (Dounce homogenization) is often used during microtubule purification by temperature-dependent cycles, anomalous results may be generated. A small number of blocks can be responsible for high resistance to drug-induced disassembly of cold-labile microtubules (Deery & Weisenberg, 1981) and can change the kinetics of Ca^{2+}-induced disassembly, causing cold-labile microtubules to be partially responsive to calmodulin (Marcum et al., 1978).

Nature of the Cold-Stabilizing Block. Experimental evidence thus far appears to implicate at least two types of polypeptides in the mechanism of cold stabilization: the 64-kilodalton switch protein, which exhibits a substantially different state of phosphorylation in cold-stable vs. cold-labile microtubules (Margolis & Rauch, 1981), and the Ca^{2+}-calmodulin binding polypeptides, referred to as STOPs, which are present in cold-stable material only. These is no evidence that the effects on cold stability produced by one or more of the STOP proteins could be attributed to nonspecific interaction of the tubulin preparation with basic proteins. Addition of stoichiometric amounts of myelin basic protein (a protein abundant in brain that is readily phosphorylated and interacts with calmodulin) had no effect on the assembly–disassembly process. Furthermore, lysozyme, which was reported by Lee et al. (1978) to increase the cold stability of microtubules, was without effect on our system at stoichiometric concentrations. The STOP proteins act at substantially substoichiometric concentrations.

We have recently succeeded in restoring cold stability to purified, cold-labile microtubules by adding back a DEAE-cellulose chromatographic fraction containing the STOP proteins (unpublished experiments). However, the exact role these proteins may play in restoring cold stability in a purified system will have to await their isolation and functional characterization.

Physiological Role of Cold Stability. The substoichiometric ratio of the switch protein and STOP polypeptides appears to fit the image of a cold-stabilizing mechanism dependent on rare microtubule blocks. There have been several examples of microtubule cold stabilization by in vitro manipulation, such as substitution of GTP by the nucleotide analogue guanosine α,β-methylenetriphosphate (Sandoval & Weber, 1980) or incubation with the plant toxin taxol (Schiff et al., 1979). Both of these agents act stoichiometrically, although the changes in tubulin conformation involved may be related to those induced by the blocking complex suggested here.

The physiological significance of cold stabilization is that it represents the removal of a microtubule subpopulation from equilibrium and from treadmilling by economical modifica-

tions. Depolymerization can be potentially controlled by protein kinases, calmodulin, or both and can be made to occur at specific sitess in response to events occurring elsewhere in the cell. Calmodulin may therefore function catalytically to depolymerize and reel in microtubules at specific disassembly sites. When chromosomes are attached to cold-stable microtubules, the consequences for control of their anaphase movement by such a disassembly process are obvious (Margolis et al., 1978; Margolis & Wilson, 1981).

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Ligand Interactions with the Solubilized Porcine Atrial Muscarinic Receptor†

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ABSTRACT: Ligand interactions with porcine atrial muscarinic receptor solubilized in a mixed-detergent system (0.4% w/v digitonin and 0.08% w/v cholate) are described. The solubilized receptor interacts with ligands in a stereospecific manner, showing about the same affinity for local anesthetics and antagonists as was found for the membrane-bound protein [Schimerlik, M. I., & Searles, R. P. (1980) *Biochemistry 19*, 3407–3413]. Agonists appear to interact with a single class of noninteracting sites that correspond to the low-affinity agonist sites in the membrane-bound preparation. Kinetic studies of L-[^{3}H]quinuclidinyl benzilate binding to the receptor indicated a two-step mechanism. The first step, in rapid preequilibrium ($K = 5.7 \times 10^{-9}$ M), was followed by a slow conformational change ($k_1 = 4 \times 10^{-3}$ s^{-1}; $k_{-1} = 1.7 \times 10^{-4}$ s^{-1}) in the receptor–ligand complex. The overall dissociation constant calculated from the association kinetics (2.3×10^{-10} M) agreed well with the thermodynamic value for K_{ov} (2.5×10^{-10} M); however, direct determination of k_{-1} gave a value about 4-fold lower (4.0×10^{-5} s^{-1}) than predicted. Possible reasons for this discrepancy are discussed.

The physiological effects of the neurotransmitter acetylcholine on the heart are thought to be mediated by (mAcChR's)[1] (Koelle, 1975). Biochemical studies of this protein have been greatly facilitated by the introduction of the affinity alkylating agent PrBCM (Young et al., 1972) as well as the radiolabeled derivative of the potent muscarinic antagonist L-QNB (Yamamura & Snyder, 1974). This membrane-bound neuroreceptor has been solubilized from brain

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[1] Abbreviations: mAcChR, muscarinic acetylcholine receptor; L-QNB, L isomer of quinuclidinyl benzilate; [^{3}H]-L-QNB, tritiated L isomer of quinuclidinyl benzilate; EDTA, ethylenediaminetetraacetic acid; PrBCM, propylbenzilylcholine mustard; PMSF, phenylmethanesulfonyl fluoride.

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