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Dynamics of Antarctic Fish Microtubules at Low Temperatures†

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ABSTRACT: The tubulins of Antarctic fishes, purified from brain tissue and depleted of microtubule-associated proteins (MAPs), polymerized efficiently in vitro to yield microtubules at near-physiological and supra-physiological temperatures (5, 10, and 20 °C). The dynamics of the microtubules at these temperatures were examined through the use of labeled guanosine 5′-triphosphate (GTP) as a marker for the incorporation, retention, and loss of tubulin dimers. Following attainment of a steady state in microtubule mass at 20 °C, the rate of incorporation of [^{3}H]GTP (i.e., tubulin dimers) during pulses of constant duration decreased asymptotically toward a constant, nonzero value as the interval prior to label addition to the microtubule solution increased. Concomitant with the decreasing rate of label incorporation, the average length of the microtubules increased, and the number concentration of microtubules decreased. Thus, redistribution of microtubule lengths (probably via dynamic instability and/or microtubule annealing) appears to be responsible for the time-dependent decrease in the rate of tubulin uptake. When the microtubules had attained both a steady state in mass and a constant length distribution, linear incorporation of labeled tubulin dimers over time occurred at rates of 1.45 s^{-1} at 5 °C, 0.48 s^{-1} at 10 °C, and 0.18 s^{-1} at 20 °C. Thus, the microtubules displayed greater rates of subunit flux, or treadmilling, at lower, near-physiological temperatures. At each temperature, most of the incorporated label was retained by the microtubules during a subsequent chase with excess unlabeled GTP. In contrast, when microtubules were assembled de novo in the presence of [α-^{32}P]GTP at 5 °C and then exposed to a pulse of [^{3}H]GTP, the ^{32}P label was lost over time during a subsequent chase with unlabeled GTP, whereas the ^{3}H label was retained. Together, these results indicate that the microtubules of Antarctic fishes exhibit, at low temperatures, behaviors consistent both with subunit treadmilling and with dynamic instability and/or microtubule annealing.

Microtubules assembled in vitro are dynamic, not static, structures. Depending upon their protein composition, the total polymer concentration, and other solution conditions, the microtubules of warm-blooded animals may exhibit subunit "treadmilling" [the net, balanced addition and loss of tubulin subunits at opposite microtubule ends (Margolis & Wilson, 1978)], "dynamic instability" [the coexistence at constant polymer mass of subpopulations of rapidly shrinking and slowly growing microtubules that interconvert rarely (Mitchison & Kirschner, 1984)], and/or end-to-end "annealing" [the joining of segments of preformed microtubules to produce longer microtubules (Rothwell et al., 1986)]. Eukaryotic cells may exploit these phenomena to modulate the assembly and stability of their microtubules in processes such as mitosis or cellular morphogenesis (Kirschner, 1980; Kirschner & Mitchison, 1986; Rothwell et al., 1986). If these processes are important physiologically, then the "cold-stable" microtubules

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of cold-adapted ectotherms should exhibit similar kinetic behaviors at low temperatures.

The brain tubulins of Antarctic fishes form microtubules efficiently in vitro at the low temperatures (−1.8 to +2 °C) experienced by these psychrophiles (Williams et al., 1985; Williams & Detrich, 1986; H. W. Detrich, III, and K. A. Johnson, unpublished results). Some of the adaptations that contribute to the facile assembly of the Antarctic fish tubulins may be present in their unique α chains (Detrich & Overton, 1986; Detrich et al., 1987). In this report, we use biochemical and electron microscopic methods to examine the dynamics of Antarctic fish microtubules at near-physiological and supraphysiological temperatures. Our results indicate that the cold-stable microtubules of these fishes display behaviors consistent both with treadmilling and with dynamic instability and/or microtubule annealing. Thus, the cytoplasmic microtubules of Antarctic fishes, like those of homeotherms at higher temperatures, are dynamic polymers.

Experimental Procedures

Materials. 1,4-Piperazinediethanesulfonic acid (Pipes),[1] EGTA, GTP (type II-S), TAME, acetyl phosphate, and acetate kinase (from *Escherichia coli*) were obtained from Sigma Chemical Co. DEAE-Sephacel was purchased from Pharmacia. [8-^{3}H]GTP (19 Ci/mmol) and [α-^{32}P]GTP (3000 Ci/mmol) were obtained from ICN Radiochemicals and from New England Nuclear, respectively.

Collection of Fishes. Specimens of the Antarctic cod, *Notothenia gibberifrons*, were collected by bottom trawling from the *R/V Polar Duke* near Low and Brabant Islands during February–March, 1988. The fishes were transported to Palmer Station, Antarctica, where they were maintained in aquaria supplied with running seawater at 1–2 °C.

Purification of Tubulin from Antarctic Fishes. Tubulin depleted of microtubule-associated proteins (MAPs) was isolated from the brain tissues of *N. gibberifrons* by a procedure involving DEAE-Sephacel chromatography and microtubule assembly (Detrich & Overton, 1986). Fish brains (50–75, 18–30 g wet weight) were homogenized in PMEGT buffer [0.1 M Pipes–NaOH (pH 6.9 at 20 °C), 1 mM $MgSO_4$, 1 mM EGTA, 0.1 mM GTP, and 2 mM TAME] at a ratio of 2.5 mL of buffer/g of tissue by means of a Tekmar Tissumizer equipped with an SDT-182EN generator (speed 40, 45 s, 0 °C). The homogenate was centrifuged (40000g, 20 min, 0 °C), and the supernatant was recovered and centrifuged again (40000g, 30 min, 0 °C). The second supernatant was recovered and applied to an ice water jacketed column (2.5-cm diameter) of PMEGT-equilibrated DEAE-Sephacel (0.67-mL bed volume of exchanger/g of original tissue). The column was then washed sequentially with PMEGT and with PMEGT–0.15 M NaCl (5 column volumes each) to remove non-tubulin proteins. Finally, tubulin was eluted with PMEGT–0.4 M NaCl, and fractions (2.5 mL) were collected at room temperature. GTP was added to each fraction to a final concentration of 1 mM, and the fractions were incubated at 20 °C for 20 min. Those fractions that developed appreciable turbidity were pooled, the pooled solution was divided into 1-mL aliquots, and microtubule polymer was collected by centrifugation (40000g, 20 min, 20 °C) of the aliquots. Pellets were stored at −70 °C. These preparations contained approximately 98% tubulin (Detrich & Overton, 1986).

Prior to use in the experiments described below, pellets were resuspended in PME buffer [0.1 M Pipes–NaOH (pH 6.9 at 20 °C), 1 mM $MgSO_4$, and 1 mM EGTA] through use of a Dounce homogenizer, and the resulting samples were centrifuged (40000g, 20 min, 0 °C) to yield solutions at final protein concentrations of ~0.5–1.2 mg/mL.

Turbidimetric Measurement of Microtubule Assembly. The polymerization of Antarctic fish tubulin was monitored turbidimetrically at 350 nm (Gaskin et al., 1974; Detrich et al., 1985) by use of a Hitachi Model 100-60 recording spectrophotometer equipped with a thermostatable cuvette chamber. Assembly reactions were initiated by rapid delivery of cold (0 °C) solutions of tubulin (in PME buffer containing 0.1 mM GTP and a GTP-regenerating system; see next section) into cuvettes prewarmed to the appropriate temperature.

Determination of Tubulin Exchange with Microtubules. To measure tubulin incorporation into and loss from microtubules at steady state in vitro, a modification of the radiolabeled GTP marker procedure of Margolis and Wilson (1978) was employed. GTP, acetyl phosphate, and acetate kinase were added to aliquots of tubulin in PME buffer (0 °C) to give final concentrations of 100 μM, 10 mM, and 0.28 units/mL, respectively. The samples (150 μL each) were warmed to the desired experimental temperatures to induce the formation of microtubules and then incubated isothermally for intervals sufficient to yield, as a minimal requirement (see Results), a steady state in polymer mass[2] (monitored by turbidimetry at 350 nm). At appropriate times postinitiation, [^{3}H]GTP (5 μCi) was added to each sample, and sample incubations were continued for the appropriate intervals. Samples were incubated in a staggered sequence so that all incubations terminated at a single time, corresponding to the start of centrifugation to collect the polymer (see below). Doubly labeled microtubules were prepared by inclusion of [α-^{32}P]GTP (~120 cpm/pmol) in the de novo assembly reaction, followed by exposure of the microtubules to a pulse of [^{3}H]GTP. In some experiments, labeled microtubules were chased by addition of unlabeled GTP (3.75 μL of a 0.1 M stock) to the samples to a final concentration of 2.6 mM. For analysis of radiolabel incorporation, microtubules were collected by centrifugation (Margolis & Wilson, 1978). Immediately prior to centrifugation, portions (100–120 μL) of the samples were layered over isothermal, 3-mL cushions of sucrose [40% (w/v) in PME] contained in centrifuge tubes. Microtubule polymer was collected by centrifugation [165000g at r_{max} (45 000 rpm), Beckman type 50 rotor, 60 min] at the appropriate temperature. The supernatant was carefully removed from each tube until approximately half remained, and the remainder was underlaid with a solution containing 50% (w/v) sucrose in PME. The fluid in each tube was then completely removed by gentle aspiration with a Pasteur pipet. Finally, each tube was rinsed twice by addition and removal of 3-mL volumes of the 40% (w/v) sucrose solution, and excess solution in the tubes was removed by means of cotton swabs. The protein pellets were dissolved in 0.5-mL volumes of 0.1 M NaOH, and samples were taken for scintillation counting and protein determinations. When microtubules were assembled de novo in the presence of [α-^{32}P]GTP, the stoichiometry of labeled

[1] Abbreviations: DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GTP, guanosine 5'-triphosphate; GXP, guanosine 5'-diphosphate or 5'-triphosphate; MAPs, microtubule-associated proteins; Pipes, 1,4-piperazinediethanesulfonic acid; TAME, *p*-tosyl-L-arginine methyl ester hydrochloride.

[2] Throughout this paper, "steady state in polymer mass" or "mass steady state" will refer to microtubule populations characterized by a stable plateau in turbidity (apparent A_{350}) and by constant sedimentable mass. These terms do not imply a priori that the microtubule populations have achieved a stable, unchanging length distribution. The latter property of a microtubule population will also be referred to as "length steady state".

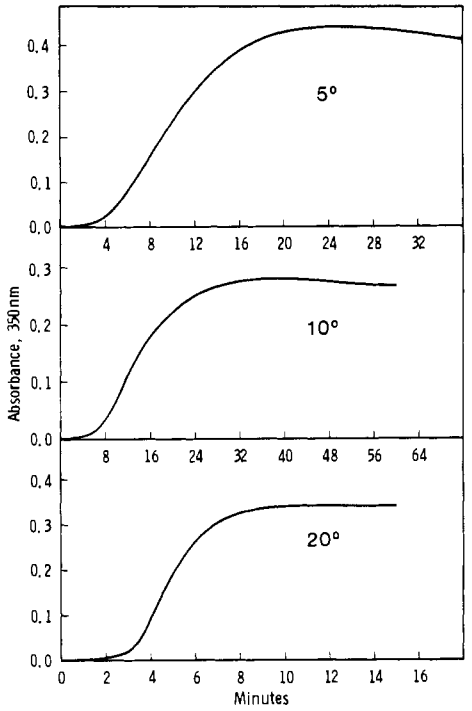

FIGURE 1: Polymerization of tubulin from an Antarctic fish at three temperatures. Samples of tubulin from *N. gibberifrons* (in PME buffer containing 0.1 mM GTP, 10 mM acetyl phosphate, and 0.28 units/mL acetate kinase) were warmed from 0 °C to final temperatures of 5, 10, or 20 °C at zero time, and microtubule assembly was monitored by turbidimetry. The development of turbidity (apparent absorbance at 350 nm) is plotted as a function of time after warming. Tubulin concentrations: 5 °C, 1.3 mg/mL; 10 °C, 0.96 mg/mL; 20 °C, 0.60 mg/mL.

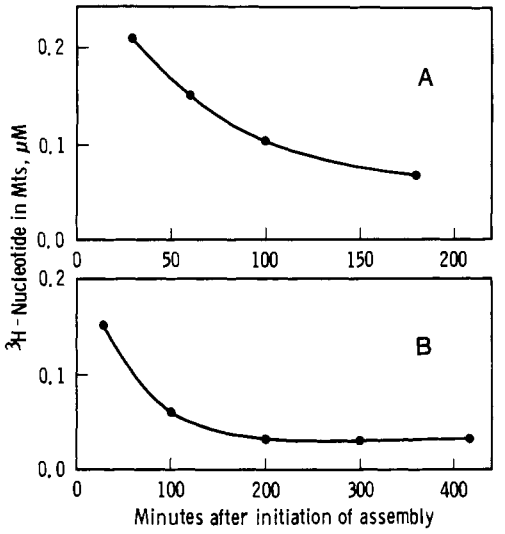

FIGURE 2: Effect of time at polymer mass steady state on the incorporation of [^{3}H]GTP-labeled tubulin dimers into microtubules during pulses of constant duration. Tubulin from *N. gibberifrons* (0.60 mg/mL in PME buffer containing 0.1 mM GTP and the GTP-regenerating system) was warmed from 0 to 20 °C at zero time to initiate microtubule assembly, and polymer mass steady state (monitored turbidimetrically) was attained 10 min postinitiation. At intervals, aliquots of the microtubule solution were exposed to pulses of [^{3}H]GTP, and microtubules were collected by centrifugation for analysis of radiolabel incorporation (see Experimental Procedures). The uptake of [^{3}H]GTP into microtubules (Mts) during pulses of 90 min (panel A) or 60 min (panel B) is plotted as a function of time after initiation of assembly (time at mass steady state = time after initiation − 10 min).

nucleotide incorporation was found to be 0.6–0.7 mol/mol of polymerized tubulin dimer.

The number of time points that can be generated by the centrifugal assay is limited by the tube capacity of the rotor. In an attempt to circumvent this limitation, the rapid filtration assay of Wilson et al. (1982) was evaluated for use with the MAP-depleted microtubules of Antarctic fishes. Buffers containing sucrose, DMSO, glycerol, and glutaraldehyde, alone or in combination, were tested for their capacity to stabilize microtubules prior to filtration. Some of the diluents did not stabilize microtubules, and others produced artifactual results [e.g., large blank values from unassembled (podophyllotoxin-poisoned) tubulin]. Thus, although it yields reliable data with MAP-containing microtubules from mammalian brain, the filtration assay was judged unacceptable for use with the MAP-depleted microtubules examined in this study. A second sedimentation assay (Farrell et al., 1987), based on the Beckman Airfuge, was not used because the equipment was not available at Palmer Station, Antarctica, the venue for most of this work.

Measurement of Microtubule Lengths. Microtubule length distributions were determined by methods similar to those of Farrell et al. (1987). Aliquots of microtubules were diluted 20-fold with PME containing 1% glutaraldehyde, drops of the fixed preparations were adsorbed to carbon-coated formvar grids of 100 and 300 mesh, and the specimens were negatively stained with aqueous uranyl acetate [1% (w/v)]. Grids were examined and photographed at magnifications of 450×–700× through use of a Philips EM300 electron microscope. Measurements of the lengths of representative samples of microtubules were made from photographic enlargements by use of a Houston Hi Pad digitizing tablet and Sigma Scan (Jandel) software. The microtubule number concentration for an experiment was calculated by dividing the concentration of tubulin in polymeric form (determined by protein assay of microtubule pellets) by the mean microtubule length and by 1690 (the number of tubulin dimers per micrometer of microtubule length). Finally, flux rates were determined by dividing the rate of [^{3}H]GTP incorporation by the microtubule number concentration.

Miscellaneous Methods. Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin as the standard. Radioactivity was determined by liquid scintillation counting in an LKB Rack Beta 1217 counter; Aquasol 2 was used as the scintillant.

Results

Microtubule Polymerization at Three Temperatures. The de novo polymerization of *N. gibberifrons* tubulin at temperatures (5, 10, and 20 °C) and protein concentrations equivalent to those used in the steady-state incorporation experiments (see below) is shown in Figure 1. The turbidity generated by the sample at 20 °C attained a stable plateau value within 10 min following the initiation of assembly. Polymerization at 5 °C and at 10 °C occurred more slowly and was accompanied by small overshoots in turbidity [cf. Detrich et al. (1985)]. Critical concentrations for microtubule assembly, estimated as the concentrations of the supernatants obtained following centrifugation (40000*g*, 20 min) of samples of the polymerized protein, were approximately 0.36, 0.17, and 0.06 mg/mL at temperatures of 5, 10, and 20 °C, respectively. Both the length distributions and the mean lengths of the microtubules, measured long after the attainment of a steady state in polymer mass, were sensitive functions of the temperature of polymerization and of the concentration of tubulin. However, at the tubulin concentrations employed in this study, mean lengths were generally greater at the lower temperatures. For example, under the conditions described in Figure 5, the mean lengths of *N. gibberifrons* microtubules were 8.4 μm at

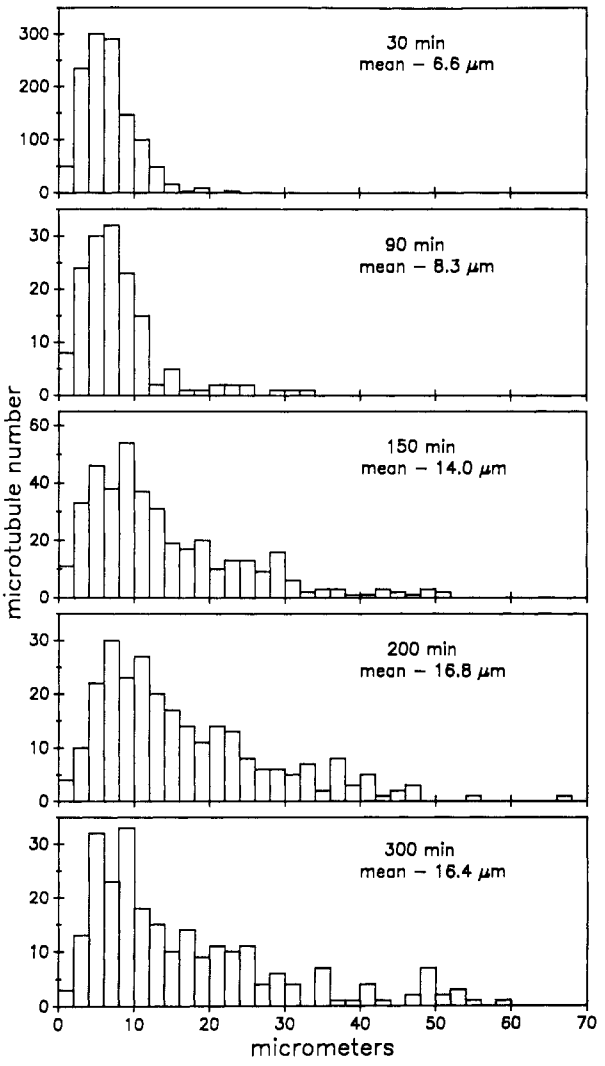

FIGURE 3: Redistribution of microtubule lengths at 20 °C. Microtubules were assembled by warming a solution of *N. gibberifrons* tubulin (0.55 mg/mL in PME buffer containing 0.1 mM GTP and the GTP-regenerating system) from 0 to 20 °C at zero time, and at intervals, aliquots were prepared for negative-stain electron microscopy. Specimens were examined, micrographs were recorded, and microtubule lengths were measured as described under Experimental Procedures. From top to bottom, the panels present histograms of microtubule number vs microtubule length for specimens prepared 30, 90, 150, 200, and 300 min after initiation of assembly. The mean length of the microtubule population at each time is noted in the appropriate panel. The number of microtubules measured for each time point was ≥150.

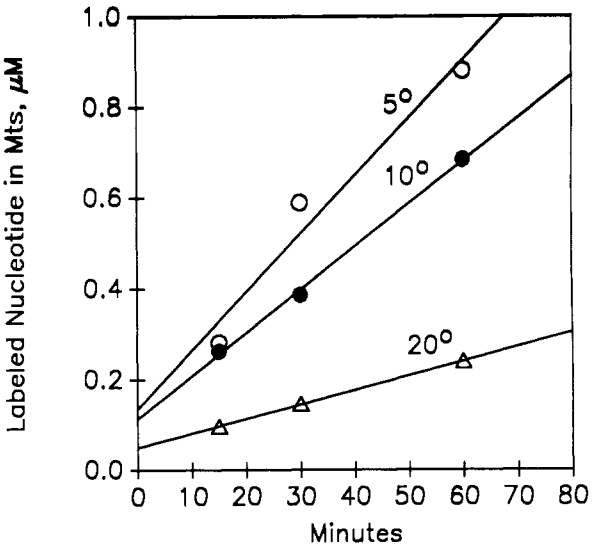

FIGURE 4: Temporal dependence of the incorporation of [^{3}H]GTP-labeled tubulin into microtubules at three temperatures. Samples of the same preparation of *N. gibberifrons* tubulin were polymerized at temperatures of 5, 10, or 20 °C for intervals (300 min at 5 and 10 °C, 220 min at 20 °C) sufficient to attain a steady state in microtubule mass and to achieve stable length distributions. The microtubules were then exposed to pulses of [^{3}H]GTP for the times indicated, collected by centrifugation, and analyzed for radiolabel incorporation (see Experimental Procedures). The uptake of radiolabeled nucleotide by the microtubules (Mts) is plotted as a function of pulse duration at 5 °C (open circles), at 10 °C (closed circles), and at 20 °C (open triangles). Tubulin concentrations: 5 °C, 1.1 mg/mL; 10 °C, 0.86 mg/mL; 20 °C, 0.59 mg/mL. Microtubule number concentrations: 5 °C, 1.5×10^{-10} M; 10 °C, 3.4×10^{-10} M; 20 °C, 3.0×10^{-10} M. The microtubule length distributions corresponding to these experiments are shown in Figure 5.

20 °C, 12.1 μm at 10 °C, and 24.8 μm at 5 °C. The increase in mean microtubule length with decreasing temperature probably results, at least in part, from reduced rates of microtubule nucleation at the lower temperatures.

Incorporation of Tubulin into Microtubules at Mass Steady State at 20 °C. Preliminary experiments were performed to evaluate the uptake of tubulin dimers, labeled with [^{3}H]GTP, as a function of time after attainment of a steady state in polymer mass (monitored turbidimetrically) at 20 °C. Figure 2A,B shows that incorporation of the labeled nucleotide during pulses of fixed duration decreased asymptotically toward a constant value as the interval that microtubules were maintained at steady state prior to label addition increased. Possible explanations for this behavior include inactivation of tubulin during long-term incubations at 20 °C, failure to attain a steady state in polymer mass that coincided temporally with the turbidity plateau, and reduction in microtubule number concentration (via rapid redistribution of microtubule lengths toward longer polymers) following attainment of a steady state in polymer mass. Experiments were performed to evaluate these possibilities. First, a sample of tubulin was polymerized by incubation at 20 °C for 190 min, the microtubules were depolymerized at 0 °C for 30 min, and the sample was incubated again at 20 °C for 30 min. Measurement of the turbidity of a second sample treated identically showed that the microtubules disassembled completely when exposed to the low temperature and that the resulting tubulin solution, when reincubated at 20 °C, polymerized to yield a plateau level of turbidity identical with that observed during its first polymerization. The amount of [^{3}H]GTP incorporated by the reassembled microtubules during a 60-min pulse was equivalent to that taken up during an identical pulse by microtubules that had only been polymerized de novo for 30 min. Together, these results indicate that the fish tubulin is not inactivated by lengthy incubation at 20 °C. Second, quantitative sedimentation analysis revealed that polymer mass was constant following attainment of plateau levels of turbidity. The enhanced uptake of labeled nucleotide at short intervals (i.e., 30 min) following initiation of assembly, therefore, does not reflect the formation of new, completely labeled microtubules. Finally, microtubule length distributions were determined at intervals following the initiation of polymerization at 20 °C. In one experiment, mean microtubule lengths increased from 6.6 μm at 30 min postinitiation to a constant value of 16.4–16.8 μm at 200–300 min postinitiation (Figure 3). Thus, the enhanced uptake of label at short intervals following initiation of polymerization probably reflects the presence of larger numbers of microtubules. Redistribution of microtubule lengths toward longer microtubules at reduced number concentration and at constant polymer mass (via dynamic instability and/or end-to-end annealing of microtubules) appears

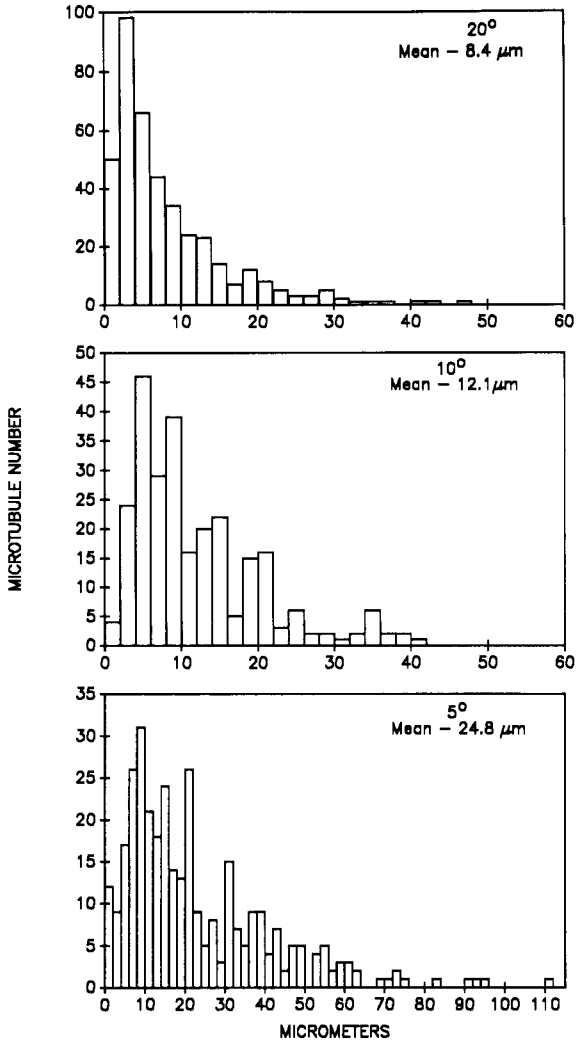

FIGURE 5: Microtubule length distributions at three temperatures. Samples of the microtubule populations from the experiments of Figure 4 were prepared for negative-stain electron microscopy 200 min after the temperature jump to 20 °C and 300 min after the temperature jumps to 5 and 10 °C (see Experimental Procedures). Specimens were examined, micrographs were recorded, and microtubule lengths were measured as described under Experimental Procedures. Each panel presents a histogram of microtubule number as a function of microtubule length. The mean length of each microtubule population is noted in the appropriate panel. The numbers of microtubules measured were 333 at 5 °C, 263 at 10 °C, and 406 at 20 °C.

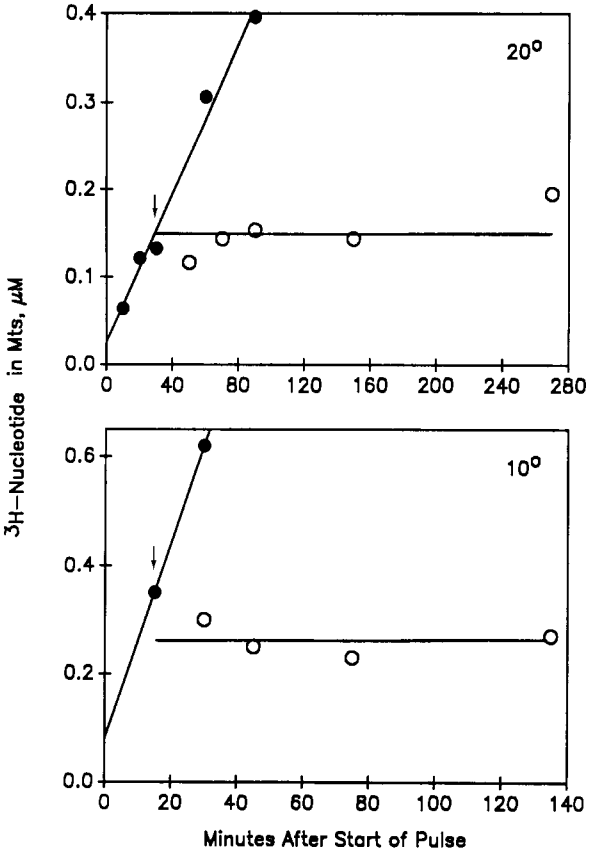

FIGURE 6: Retention of labeled guanine nucleotide by microtubules during a chase with unlabeled GTP. Microtubules were assembled from *N. gibberifrons* tubulin at 20 °C (top panel, protein concentration = 0.55 mg/mL) or at 10 °C (bottom panel, protein concentration = 1.0 mg/mL). After attainment of constant polymer mass and stable length distributions (210 min postinitiation at 20 °C, 220–340 min postinitiation at 10 °C), microtubule samples were exposed to pulses of [^{3}H]GTP of varying duration, and the microtubules were collected by centrifugation and analyzed for radionucleotide incorporation. Following pulses of 30 min at 20 °C and 15 min at 10 °C, additional samples of labeled microtubules were chased for varying intervals by addition of a 26-fold excess of unlabeled GTP (arrows), and the microtubules were collected and analyzed for retention of the label (see Experimental Procedures). At each temperature, radionucleotide incorporation into microtubules (Mts) during the pulse (closed circles) and retention by microtubules during the chase (open circles) are plotted as functions of time following the start of the pulse.

to be responsible for the reduced uptake of label by microtubule polymer exposed to pulses of [^{3}H]GTP at longer intervals postinitiation.

In all subsequent experiments, samples of tubulin were incubated at mass steady state for intervals sufficient to achieve stable length distributions prior to addition of [^{3}H]GTP. Furthermore, with the exception of the pulse–chase experiment at 10 °C (Figure 6, bottom panel), *each* sample in a given experiment was incubated for the *same* interval prior to exposure to the pulse of labeled nucleotide.

Dynamics of Tubulin Exchange with Microtubules following Attainment of Stable Polymer Length Distributions. Figure 4 shows a comparison of the initial rates of [^{3}H]GTP uptake into microtubules at 5 °C, at 10 °C, and at 20 °C under conditions of constant polymer mass and stable length distribution. Figure 5 presents the length distributions of the same three microtubule populations. Incorporation of label at each temperature was a linear function of time, and the greatest rate of uptake occurred at 5 °C, the temperature at which the mean microtubule length was greatest and the microtubule number concentration was smallest. The linear uptake of [^{3}H]GTP is consistent with tubulin incorporation into microtubules by a treadmilling mechanism. The corresponding flux rates were 1.45 s^{-1} (3 μm/h) at 5 °C, 0.48 s^{-1} (1 μm/h) at 10 °C, and 0.18 s^{-1} (0.4 μm/h) at 20 °C. Figure 4 also shows that at each temperature a "burst" of [^{3}H]GTP incorporation, indicated by the nonzero y intercepts of the best-fitting straight lines through each set of data points, was observed. Similar bursts reported for microtubules from bovine brain have been attributed to nucleotide exchange at microtubule ends and/or to rapid transitions between shrinking and growing phases at microtubule ends (Farrell et al., 1987).

Pulse–chase experiments were performed to investigate further the mechanism responsible for label incorporation into microtubules at mass and length steady state. When a 26-fold excess of unlabeled GTP was added to microtubules at 20 °C following a 30-min pulse of [^{3}H]GTP (Figure 6, upper panel, arrow), label was retained by the microtubules (open circles) for an additional 240 min. Retention of label during the chase is consistent with the treadmilling mechanism and indicates that the slow, linear phase of radionucleotide incorporation (Figure 4; Figure 6, closed circles) does not result from an

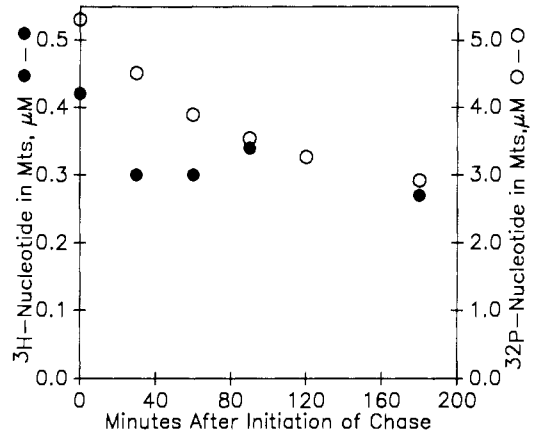

FIGURE 7: Dynamics of radionucleotide exchange with doubly labeled microtubules. Tubulin (1.1 mg/mL) from *N. gibberifrons* was polymerized for 300 min at 5 °C in the presence of [α-^{32}P]GTP. Samples of the [^{32}P]GXP-labeled microtubules were exposed to 30-min pulses of [^{3}H]GTP and then chased for varying intervals by addition of a 26-fold excess of unlabeled GTP. The microtubules were collected by centrifugation and analyzed for retention of the two labels (see Experimental Procedures). Retention of the ^{32}P and ^{3}H labels by the microtubules (Mts) is plotted as a function of time after initiation of the chase.

equilibrium exchange mechanism (Zeeberg et al., 1980). Similarly, most of the label incorporated into steady-state microtubules at 10 °C was retained for an interval at least 8 times the 15-min pulse duration (Figure 6, lower panel). The small drop in incorporated label observed at the start of the chase (see arrows), a phenomenon also reported for mammalian microtubules (Farrell et al., 1987), is probably attributable to the same mechanism(s) that produce(s) the burst of label uptake noted previously (Figure 4).

Finally, tubulin addition to and loss from microtubules at mass and length steady state was evaluated by means of a double-label protocol. Microtubules were assembled at 5 °C in the presence of [α-^{32}P]GTP for an interval sufficient to produce a stable length distribution, exposed to a pulse of [^{3}H]GTP, and then chased by addition of unlabeled GTP. During the chase, the ^{32}P label was lost slowly, while the ^{3}H label was retained (following an initial burst of label loss), by the microtubules (Figure 7). Although the rate of ^{32}P loss was not linear over the interval examined, the limiting values for the rate of loss, ~0.025 and ~0.008 μM min^{-1} (measured 0–60 and 60–180 min, respectively, after the start of the chase), were approximately equivalent to the rate of ^{3}H uptake, 0.014 μM min^{-1}, during the pulse. Thus, at polymer mass steady state, tubulin dimers appear to add and to dissociate in a balanced fashion at the opposite ends of *N. gibberifrons* microtubules at temperatures near the physiological.

Discussion

The results presented here demonstrate that brain microtubules from Antarctic fishes are dynamic polymers at temperatures between 5 and 20 °C. Following polymerization in vitro to a steady state in polymer mass, the microtubules undergo a rapid redistribution of their lengths, probably via dynamic instability and/or microtubule annealing, until a stable population length distribution is achieved. Concomitant with the decrease in microtubule number concentration, the rate of incorporation of tubulin dimers decreases asymptotically to a constant value. Subsequently, the microtubules incorporate, retain, and lose tubulin dimers (labeled with [^{3}H]GXP) by a mechanism consistent with treadmilling. Both the retention of labeled GXP by microtubules during the pulse–chase experiments (Figure 6) and the differential loss of label during the chase of doubly labeled microtubules (Figure 7) provide compelling support for the latter conclusion. Thus, different dynamic behaviors appear to predominate at different stages of microtubule assembly.

The MAP-depleted microtubules of *N. gibberifrons* displayed the greatest rate of steady-state subunit flux, 1.45 s^{-1} or 3 μm/h, at a temperature, 5 °C, close to the natural habitat temperatures (−1.8 to +2 °C) of this fish. Furthermore, the *trend* of the data (an 8-fold increase in flux rate as the temperature decreased from 20 to 5 °C) suggests that the treadmilling rate may be greater yet at temperatures in the physiological range. The increased rates of flux at low temperatures presumably result from a differential effect of temperature on the magnitudes of the rate constants governing tubulin addition and loss at the two ends of a microtubule. By contrast, the limited data available for MAP-rich mammalian microtubules suggest that subunit flux rates at steady state are relatively invariant (0.3–0.45 s^{-1}, 0.7–1 μm/h) in the temperature range 30–37 °C (Margolis & Wilson, 1978; Farrell & Jordan, 1982; Farrell et al., 1987; Hotani & Horio, 1988), and the temperature dependence of treadmilling by MAP-depleted mammalian microtubules has apparently not been investigated. Interestingly, the steady-state flux rate of *N. gibberifrons* microtubules at 5 °C, 1.45 s^{-1}, is somewhat larger than the values (0.3–0.45 s^{-1}) obtained for the MAP-rich microtubules of mammals at near-physiological body temperatures (30–37 °C) but is smaller than the flux rates (24.6–27.6 s^{-1}, ~52–59 μm/h) (Farrell et al., 1987; Cote & Borisy, 1981) for MAP-depleted mammalian microtubules at 37 °C. Perhaps the tubulins of Antarctic fishes have evolved modifications that confer an intermediate rate of dimer flux through microtubules in the absence of MAPs. (The effect of brain MAPs from Antarctic fishes on the treadmilling of the piscine microtubules remains to be determined.) Therefore, when examined at physiologically appropriate temperatures, MAP-depleted microtubules both from a cold-adapted ectotherm and from a homeotherm treadmill in vitro, but not at identical rates. Conservation of microtubule treadmilling by cold- and warm-blooded organisms suggests that treadmilling may play an important physiological role in eukaryotic cells.

Microtubules at mass steady state in vitro exhibit a variety of dynamic behaviors, including treadmilling, dynamic instability, and end-to-end annealing. Depending upon the experimental conditions, MAP-free microtubules have been reported to exchange tubulin dimers by treadmilling (Farrell et al., 1987), by dynamic instability (Horio & Hotani, 1986; Kristofferson et al., 1986; Walker et al., 1988), or by a combination of the two processes (Farrell et al., 1987). When incorporated into microtubules, MAPs suppress both treadmilling and dynamic instability (Farrell et al., 1987; Hotani & Horio, 1988), yielding polymers characterized by slow subunit flux rates (Margolis & Wilson, 1978; Farrell & Jordan, 1982) and few, if any, transitions between shrinking and growing phases at their ends (Hotani & Horio, 1988). Redistribution of microtubule lengths at mass steady state appears to result both from the efficient annealing of short polymers (Caplow et al., 1986; Rothwell et al., 1986, 1987; Williams & Rone, 1988) and from subunit exchange between polymers mediated by dynamic instability (Mitchison & Kirschner, 1984; Kristofferson et al., 1986; Rothwell et al., 1987). Our results, together with those of Farrell et al. (1987), indicate that the relative contributions of treadmilling, dynamic instability, and probably annealing to the dynamics of microtubules may change following attainment of polymer mass steady state. We conclude that no single reaction pathway

can explain the solution dynamics of all microtubule systems at all times and under all conditions.

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Discovery and Mapping of Discrete Binding Sites on Nucleosome Core Particles for a Photoaffinity Derivative of Spermine†

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ABSTRACT: A polyamine photoaffinity analogue, (azidonitrobenzoyl)spermine (ANB-spermine), has been synthesized and characterized. Thermal denaturation studies showed that ANB-spermine interacted noncovalently with nucleosome core particles similarly to naturally occurring polyamines carrying the same charge (+3). Photoaffinity labeling of core particles with ANB-spermine resulted in covalent binding of about 3% to the DNA and a similar amount to the histones. The locations of covalent binding to DNA were determined by exonuclease protection using T4 DNA polymerase–exonuclease. There was significant nonspecific binding and 7 discrete sites of preferred binding: at the 3′ end and centered at positions 15, 36, 56, 86, 109 and 130 bases from the 3′ end. A possible site at the 5′ end could not be determined by this technique. The sites were roughly symmetrical about the center of the DNA molecule, indicating labeling of both strands of the helix. The sites were offset from DNase I nicking sites by 3–5 bases, indicating that they are not on the exterior of the core particle. The sites occurred in the core particle where the two turns of the DNA supercoil are closely apposed, with a conspicuous gap where there is a single turn of the supercoil. By use of a CTAB/SDS–protein two-dimensional gel system, it was found that histones were labeled to differing extents, with H3 = H2B > H2A > H4. Partial tryptic digestion showed that the histones were labeled mainly (73–80%) in the central, globular regions. The data indicate that ANB-spermine and, presumably, the naturally occurring polyamines bind both in a nonlocalized fashion and at specific sites on the nucleosome core particle.

Chromatin undergoes major alterations during the transitions from the inactive to the potentially active to the actively transcribed state (Reeves, 1988; Garrard et al., 1988) and during replication (DePamphilis & Wassarman, 1980; Annunziato & Seale, 1983). These alterations must fundamentally open up the compact form of bulk chromatin and allow the bases of the DNA to become accessible to base pairing with the nascent RNA or DNA. Acetylation of histones has been correlated with transcription and replication (Allfrey et al., 1964; Allfrey, 1977; Allegra et al., 1987; Matthews, 1988; Hebbes et al., 1988). Experiments have shown that histone

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