Polymerization of Antarctic Fish Tubulins at Low Temperatures: Energetic Aspects†

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ABSTRACT: Tubulins were purified from the brain tissues of three Antarctic fishes, *Notothenia gibberifrons*, *Notothenia coriiceps neglecta*, and *Chaenocephalus aceratus*, by ion-exchange chromatography and one cycle of temperature-dependent microtubule assembly and disassembly in vitro, and the functional properties of the protein were examined. The preparations contained the α- and β-tubulins and were free of microtubule-associated proteins. At temperatures between 0 and 24 °C, the purified tubulins polymerized readily and reversibly to yield both microtubules and microtubule polymorphs (e.g., "hooked" microtubules and protofilament sheets). Critical concentrations for polymerization of the tubulins ranged from 0.87 mg/mL at 0 °C to 0.02 mg/mL at 18 °C. The van't Hoff plot of the apparent equilibrium constant for microtubule elongation at temperatures between 0 and 18 °C was linear and gave a standard enthalpy change ($\Delta H°$) of +26.9 kcal/mol and a standard entropy change ($\Delta S°$) of +123 eu. At 10 °C, tubulin from *N. gibberifrons* polymerized efficiently at high ionic strength; the critical concentration increased monotonically from 0.041 to 0.34 mg/mL as the concentration of NaCl added to the assembly buffer was increased from 0 to 0.4 M. Together, the results indicate that the polymerization of tubulins from the Antarctic fishes is entropically driven and suggest that an increased reliance on hydrophobic interactions underlies the energetics of microtubule formation at low temperatures. Thus, evolutionary modification to increase the proportion of hydrophobic interactions (relative to other bond types) at sites of interdimer contact may be one adaptive mechanism that enables the tubulins of cold-living poikilotherms to polymerize efficiently at low temperatures.

The polymerization of cytoplasmic microtubules from their functional subunits, tubulin $\alpha\beta$ dimers and microtubule-associated proteins (MAPs),[1] is an entropically driven process mediated largely by the displacement of structured water at sites of intersubunit contact (Correia & Williams, 1983). Because entropy drives the polymerization, microtubule formation is energetically favored as temperature increases. Thus, the "cold-labile" microtubules of warm-blooded animals assemble from their subunits at physiological temperatures (30–37 °C) and depolymerize at lower temperatures (0–4 °C). In contrast, the cytoplasmic microtubules of the Antarctic fishes, a group of cold-living poikilotherms that diverged from temperate osteichthyans approximately 40 million years ago as the Antarctic seas cooled (DeWitt, 1971), must assemble in an unfavorable thermal environment (−1.8 to +2 °C). Presumably, the Antarctic fishes have evolved microtubule proteins with modifications that favor microtubule polymerization at their low habitat temperatures.

The capacity to form microtubules at low temperatures may reflect adaptive changes in the tubulin subunits, evolution of microtubule-associated, cold-stabilizing factors, or both. Recent evidence suggests that the first possibility is most likely. For example, the tubulins of temperate and cold-adapted poikilotherms polymerize efficiently at body temperatures well below those of homeotherms. The critical concentrations for assembly of microtubules by purified, MAP-free tubulins from eggs of a sea urchin (*Strongylocentrotus purpuratus*) and of the surf clam (*Spisula solidissima*) are small (0.71–0.81 mg/mL for the urchin and 0.36 mg/mL for the clam) at the respective habitat temperatures (15–18 and 22 °C) of these organisms (Suprenant & Rebhun, 1983, 1984; Detrich et al., 1985a). Similarly, Williams et al. (1985) have shown that near-homogeneous brain tubulin from *Pagothenia borchgrevinki* has a critical concentration of 0.74 mg/mL at −1.8 °C, the habitat temperature of this Antarctic fish. For comparison, under similar buffer conditions, the assembly of microtubules from purified mammalian brain tubulins requires elevated temperatures (37 °C) and high protein concentrations [>2.5 mg/mL in the absence of glycerol or dimethyl sulfoxide (Herzog & Weber, 1977; Himes et al., 1977)]. These results suggest that the biochemical adaptations that enable the microtubules of poikilotherms to form at low temperatures reside in their tubulin subunits.

Our long-range objectives are to determine the structural adaptations and polymerization energetics of the tubulins of Antarctic fishes. We have shown previously that the brain tubulins of Antarctic fishes differ structurally from temperate fish and mammalian tubulins (Detrich & Overton, 1986, 1988) and that many, if not most, of the differences are present in the α chains (Detrich et al., 1987a). In this report, we characterize the temperature dependence of the polymerization of MAP-free brain tubulins from Antarctic fishes, and we describe the structure of the polymer formed in vitro. We also examine for the first time the salt dependence of microtubule formation by the tubulins of these cold-adapted fishes. Finally,

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[1] Abbreviations: DEAE, diethylaminoethyl; DTE, dithioerythritol; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; MAP, microtubule-associated protein; Pipes, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; TAME, *p*-tosyl-L-arginine methyl ester hydrochloride.

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we describe an improved protocol for the rapid purification of homogeneous Antarctic fish brain tubulins with yields that are substantially larger (~10-fold) than that obtained by two cycles of temperature-dependent microtubule assembly/disassembly followed by phosphocellulose chromatography (Williams et al., 1985). Our results indicate that polymerization of Antarctic fish tubulins is entropically driven and that the contribution of hydrophobic interactions to the energetics of assembly of these tubulins at low temperatures is significantly greater than was previously appreciated [cf. Williams et al. (1985)]. Apparently, the assembly-enhancing adaptations present in the tubulins of Antarctic fishes include increases in the proportion or in the strength (or both) of the hydrophobic interactions that form at sites of interdimer contact. Preliminary reports of some of this work have appeared (Detrich et al., 1985b, 1987b; Williams & Detrich, 1986).

Experimental Procedures

Materials. Pipes, EGTA, GTP (type II-S), and TAME were obtained from Sigma. DEAE-Sephacel was supplied by Pharmacia, Inc. Acrylamide (>99.9%) and *N,N'*-methylenebis(acrylamide) were products of Bio-Rad Laboratories, and urea (enzyme grade) was purchased from Bethesda Research Laboratories. Araldite 502, glutaraldehyde, osmium tetroxide, sodium cacodylate, and uranyl acetate were obtained from Ted Pella, Inc. Tannic acid was supplied by Mallinckrodt. Other chemicals were reagent grade.

Collection of Fishes. Specimens of the Antarctic fishes were collected by bottom trawling from the *R/V Polar Duke* near Low and Brabant Islands in the Palmer Archipelago. The fishes were transported to Palmer Station, Antarctica, and were maintained in aquaria supplied with running seawater (1–2 °C).

Preparation of Tubulins from Antarctic Fishes. Tubulins were purified from the brain tissues of three Antarctic fishes (*Notothenia gibberifrons*, *Notothenia coriiceps neglecta*, and *Chaenocephalus aceratus*) by DEAE ion-exchange chromatography and one cycle of microtubule assembly as described previously (Detrich & Overton, 1986). Three minor modifications were introduced in later preparations: (1) EGTA and GTP (final concentrations of 1 and 0.1 mM, respectively) were added to the buffer (0.1 M Pipes–NaOH, 1 mM $MgSO_4$, and 1 mM TAME, pH 6.9 at 20 °C) used for homogenization of brain tissue; (2) $CaCl_2$ was not added to brain homogenates; and (3) EGTA (final concentration 1 mM) was added to the buffers employed for DEAE-Sephacel ion-exchange chromatography. These modifications increased the maximal yield of tubulin [typically 1.2 mg/g (wet weight) of fish brain tissue] by 15–20% with respect to the original protocol (Detrich & Overton, 1986) without altering the functional properties of the protein. The purified tubulins were stored at −70 °C as microtubule pellets.

Experimental Buffer Systems. For turbidimetry and electron microscopy, tubulin was polymerized in PME buffer (0.1 M Pipes–NaOH, 1 mM $MgSO_4$, and 1 mM EGTA, pH 6.82 at 20 °C) containing GTP (1 mM). Critical concentrations were measured in PMG buffer (0.1 M Pipes–NaOH, 1 or 2 mM $MgSO_4$, and 1 mM GTP, pH 6.82 at 20 °C) supplemented with $CaCl_2$, EGTA, and DTE or DTT. The modifications and additions to these buffer systems are described below and in the appropriate figure legends.

Turbidimetric Measurement of Assembly. Microtubule assembly was monitored turbidimetrically (Gaskin et al., 1974; Detrich et al., 1985a) at 350 nm by means of a Hitachi Model 100-60 recording spectrophotometer equipped with a four-cell thermostatable cuvette carousel. The assembly of microtubule polymer was initiated by three different methods: (1) rapid warming of GTP-containing solutions of tubulin initially at 0 °C; (2) addition of EGTA to solutions of tubulin containing calcium ion; or (3) addition of GTP to solutions of tubulin lacking the nucleotide.

Determination of Critical Concentrations. Critical concentrations for the polymerization of Antarctic fish tubulins at temperatures between 0 and 18 °C were determined by a modification of the quantitative sedimentation assay of Johnson and Borisy (1975). This procedure yields the steady-state distribution of assembled microtubule protein in terms of two operationally defined species: polymer (microtubules) and monomer (the polymerizing unit). Briefly, pellets of microtubules were resuspended in PMG buffer containing $CaCl_2$ (5 or 8 mM) (and, in some cases, 1 mM DTE) to produce a suspension at a protein concentration of 2–3 mg/mL. The resuspended microtubules were incubated at 0 °C for 30 min and then centrifuged at 1–2 °C (30000*g*, 20 min) to remove undissociated material. The supernatant (containing tubulin subunits) was recovered, excess EGTA (final concentrations of 6.2 or 10 mM, respectively, for the two concentrations of $CaCl_2$ employed) was added to sequester the calcium ion, and the solution was incubated at 20 or 24 °C for 60 min. Following polymerization, aliquots of the microtubule suspension were diluted with isothermal buffer of identical composition to produce sample sets containing five different total protein concentrations. Each set of samples was then incubated at the desired experimental temperature for 30–45 min, an interval sufficient to attain a new steady-state level of polymerization as judged by turbidimetry. Each sample set was centrifuged (30000*g*, 20 min) at the appropriate temperature, and the supernatants were recovered to yield the monomer fraction. Protein concentrations of the monomer fractions were determined as described below. The critical concentration at each temperature was estimated as the *y* intercept of the linear regression line through the monomer (supernatant) data points (neglecting those data points obtained at total protein concentrations that did not yield detectable polymer pellets). Sedimentation of tubulin depended specifically upon the formation of microtubule polymer; tubulin was recovered quantitatively (102.8 ± 0.6%) in the supernatant following incubation with 10^{-4} M colchicine and subsequent centrifugation.

The critical concentrations for assembly of *N. gibberifrons* tubulin at elevated ionic strengths were determined at 10 °C by the method outlined in the previous paragraph. Stock solutions of the tubulin were prepared in PMG buffer ([$MgSO_4$] = 2 mM) containing 8 mM $CaCl_2$, 1 mM DTT, and concentrations of NaCl from 0 to 0.6 M. EGTA was added to each stock to a final concentration of 10 mM, and the solutions were incubated at 24 °C for 70 min. Following polymerization, each stock was diluted with buffer containing the appropriate concentration of NaCl to produce a set of five samples differing in their total protein concentrations. The sample sets were incubated at 10 °C for 45 min and then centrifuged (30000*g*, 20 min, 10 °C) to separate the polymer and monomer fractions. The protein concentrations of the supernatant (monomer) fractions were measured (see below), and critical concentrations were determined as before. Greater than 94% (94.6 ± 1.1%) of the protein remained in the supernatant when samples of each tubulin stock were incubated in the presence of 10^{-4} M colchicine prior to centrifugation. Thus, sedimentation of protein was dependent upon the as-

sembly of microtubule polymer.

Electron Microscopy. Microtubule samples for negative-stain electron microscopy were polymerized both at physiological (0 °C) and at nonphysiological (20 °C) temperatures. Microtubules assembled at 0 °C were fixed with aqueous glutaraldehyde [0.8% (w/v)] for 10 min at 0 °C. Following fixation, single drops of the sample were applied to collodion- or formvar-coated grids for 20 s, excess solution was withdrawn with filter paper, and the grids were rinsed with two drops of water. Excess water was drawn off with filter paper, and the samples were stained with one drop of 2% (w/v) aqueous uranyl acetate for 15–20 s. Finally, the uranyl acetate solution was removed with filter paper, and the grids were allowed to dry at room temperature. Microtubules polymerized at 20 °C were prepared for negative-stain electron microscopy without prior fixation by a modification of the method of Valentine et al. (1968).

For thin-section electron microscopy, microtubule polymer was fixed in a solution containing glutaraldehyde [0.25% (w/v)] and tannic acid [6% (w/v)] in 50 mM sodium phosphate buffer (pH 6.8) for 90 min at 18 °C [cf. Pierson et al. (1978)]. Following centrifugation (30000*g*, 30 min, 18 °C) to collect the fixed polymer, the microtubule pellet was rinsed once with 50 mM sodium phosphate buffer (pH 6.8), postfixed in 1% (w/v) OsO_4 in 0.1 M sodium cacodylate (pH 7.2) for 60 min, dehydrated through an acetone series, and embedded in Araldite. Thin sections were stained with alcoholic uranyl acetate followed by lead citrate (Pierson et al., 1978; Kim et al., 1979).

Samples were observed by means of Zeiss EM 10 or Philips EM 300 electron microscopes operated at 80 kV.

Dark-Field Light Microscopy. Microtubule polymer was visualized by dark-field light microscopy as described by Suprenant and Dentler (1982). Samples were observed by means of a Zeiss Standard 16 microscope equipped with a Zeiss oil-immersion ultracondenser (1.2/1.4 N.A.), a Zeiss 40× Planachromat objective lens (0.65 N.A.), and a 75-W xenon burner (Zeiss). Specimen temperature was controlled through use of a water-jacketed stage platform connected to a Haake A-82 refrigerating circulator bath. Temperatures were measured directly from the microscope slides by a YSI thermocouple thermometer equipped with a surface temperature probe.

Electrophoresis and Isoelectric Focusing. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) on slab gels containing linear gradients of acrylamide [4–16% (w/v)] and of urea (1–8 M) (Kim et al., 1979; Detrich et al., 1985a). High-resolution isoelectric focusing was performed by a modification (Detrich & Overton, 1986) of the method of Field et al. (1984). Gels were fixed, stained with Coomassie Brilliant Blue R-250, and destained as described previously (Detrich & Wilson, 1983; Detrich et al., 1985a; Detrich & Overton, 1986).

Protein Determinations. Protein concentrations were measured by the method of Bradford (1976) or, occasionally, by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

Results

Purification of Tubulins. Tubulins were prepared from the brain tissues of three species of Antarctic fishes by DEAE ion-exchange chromatography and one cycle of microtubule assembly in vitro. Figure 1 presents an SDS/urea–polyacrylamide gradient gel containing protein fractions obtained during purification of tubulin from *N. gibberifrons*. Clearly, proteins corresponding to the α- and β-tubulins were selectively

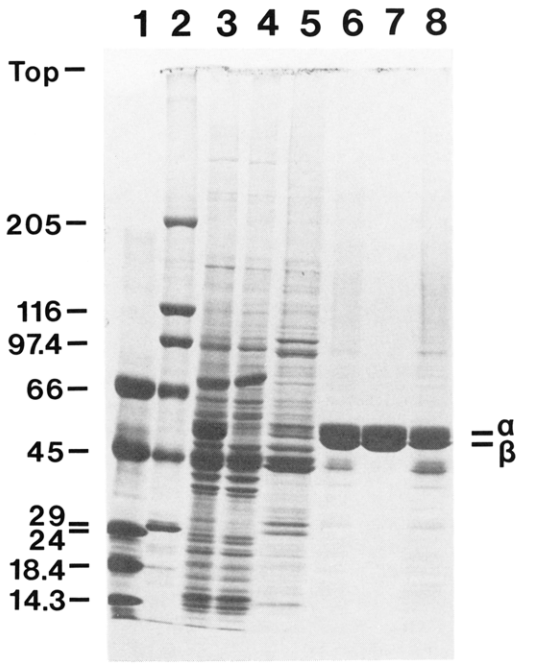

FIGURE 1: Purification of brain tubulin from the Antarctic fish *Notothenia gibberifrons*. Tubulin was purified from brain tissue by DEAE ion-exchange chromatography and one round of microtubule polymerization as described under Experimental Procedures. Protein fractions obtained at various stages of the purification were examined by electrophoresis on an SDS/urea–polyacrylamide gradient gel. Lane 1, low molecular weight standards, including bovine serum albumin (66 000), ovalbumin (45 000), trypsinogen (24 000), β-lactoglobulin (18 400), and lysozyme (14 300); lane 2, high molecular weight standards, including myosin (205 000), β-galactosidase (116 000), phosphorylase *b* (97 400), bovine serum albumin, ovalbumin, and carbonic anhydrase (29 000); lane 3, high-speed supernatant from *N. gibberifrons* brain; lane 4, proteins present in the flow-through fraction following application of the high-speed supernatant to a column of DEAE-Sephacel; lanes 5 and 6, proteins released from the ion-exchange resin by PMTG buffer (0.1 M Pipes–NaOH, 1 mM $MgSO_4$, 1 mM TAME, and 0.1 mM GTP, pH 6.9 at 20 °C) containing 0.15 and 0.40 M NaCl, respectively; lanes 7 and 8, pellet and supernatant obtained by polymerization of sample 6 (20 °C, 20 min) followed by centrifugation to collect the microtubule polymer. The molecular weights of the standards ($\times 10^{-3}$) and the positions of the tubulin chains (α and β) and the top of the gel are indicated on the vertical axes.

removed from the high-speed supernatant following adsorption of the supernatant to DEAE-Sephacel [compare lanes 3 (high-speed supernatant) and 4 (proteins not bound by the ion-exchange resin)]. Application of column buffer containing 0.15 M NaCl released a fraction composed largely of non-tubulin proteins (lane 5). Finally, a fraction enriched in tubulin (lane 6) was eluted with column buffer containing 0.4 M NaCl. Following assembly of the tubulin-enriched material and sedimentation to collect the polymer, the pelleted protein (lane 7) consisted of the α- and β-tubulins and was free of detectable MAPs; non-tubulin proteins were recovered quantitatively in the supernatant (unpolymerized) fraction (lane 8). The yield of tubulin (1.2 mg/g of brain tissue) realized by this method was substantially greater than the quantity (0.12 mg/g of brain tissue) obtained from another Antarctic fish, *P. borchgrevinki*, by a protocol involving two cycles of temperature-dependent microtubule assembly/disassembly in vitro and phosphocellulose chromatography (Williams et al., 1985). Furthermore, this DEAE/assembly-purified tubulin did not contain the non-tubulin contaminants present in the cycle- and phosphocellulose-purified tubulin preparation (Williams et al., 1985).

Assembly of Microtubules. When a solution containing purified, MAP-free tubulin from *N. gibberifrons* (0.5 mg/mL

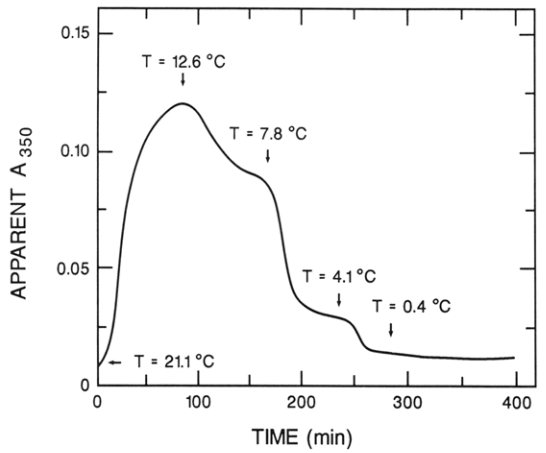

FIGURE 2: Reversible polymerization and depolymerization of *N. gibberifrons* tubulin. A sample of purified tubulin (0.5 mg/mL in PME buffer containing 1 mM GTP) was warmed from 0 to 21.1 °C at zero time, and the turbidity generated by the sample was observed spectrophotometrically (apparent A_{350}). At intervals (see arrows), the temperature of the sample was reduced to the indicated values.

in PME plus 1 mM GTP) was warmed from 0 to 21.1 °C, polymers formed as judged by turbidimetry (Figure 2). Following the temperature jump, solution turbidity increased and approached a plateau value. Numerous filaments were observed in this solution by dark-field light microscopy (data not shown). As the sample temperature was reduced (Figure 2, see arrows specifying times of the temperature shifts), the turbidity of the solution decreased progressively, approaching a new plateau value at each temperature. Upon cooling to 0.4 °C, the turbidity of the sample returned almost to the value observed at zero time. These results are consistent with the reversible polymerization of *N. gibberifrons* tubulin in response to changes in temperature.

Figures 3A and 4 show representative electron micrographs of the polymers formed when solutions of Antarctic fish tubulins were warmed to 18–20 °C. Microtubules of normal morphology were the predominant product of assembly (Figure 3A), although some sheets, microtubule–sheet hybrids, and "hooked" microtubules were also found (Figure 4); the polymorphs may be transient intermediates on the pathway to formation of microtubules [cf. Detrich et al. (1985a)]. Similarly, when assembly was initiated at 0 °C by addition of GTP to a nucleotide-free solution of *N. gibberifrons* tubulin (4.4 mg/mL in PME buffer), microtubules constituted the majority of the polymer observed by negative-stain electron microscopy (Figure 3B). The microtubules formed by the three fish tubulins appeared to be identical in structure. Thus, under the conditions employed in this study, the tubulins of Antarctic fishes assemble to form microtubules in vitro, at temperatures ranging from 0 to 20 °C.

We also examined the effects of GTP, calcium ion, and antimitotic drugs (colchicine, podophyllotoxin) on the polymerization of Antarctic fish tubulins. As shown in Figure 5, polymerization of *N. gibberifrons* tubulin at 20 °C was inhibited in solutions lacking free GTP (compare samples 1 and 2). Simultaneous addition of GTP (1 mM) and calcium ion (10 mM) to the nucleotide-free sample (see arrow, sample 2) produced a small increase in solution turbidity. When excess EGTA was added to this sample, its turbidity increased rapidly to a plateau value 24% greater than that attained by the control (sample 1). Thus, GTP supports, and calcium ion inhibits, the formation of microtubule polymer by Antarctic fish tubulins. Colchicine at a concentration of 10^{-4} M prevented the polymerization of *N. gibberifrons* tubulin at temperatures

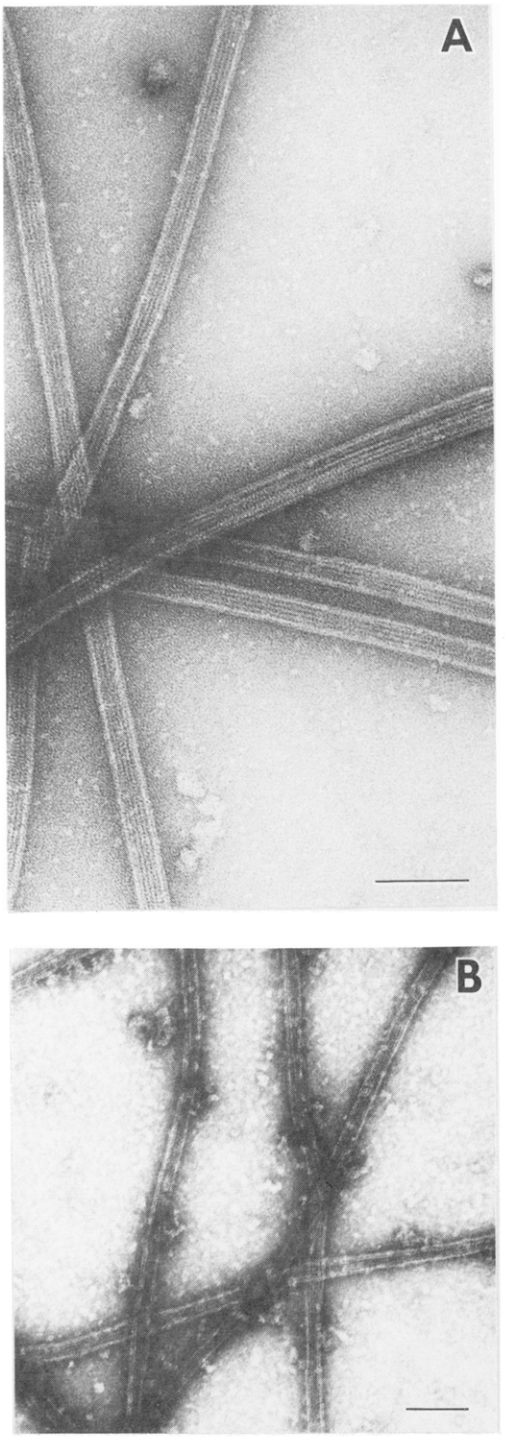

FIGURE 3: Electron micrographs of microtubule polymer assembled in vitro from Antarctic fish tubulins. (A) Polymerization at elevated, nonphysiological temperature. A solution of *N. coriiceps neglecta* tubulin (0.64 mg/mL in PME buffer containing 1 mM GTP) was warmed from 0 to 20 °C at zero time, and a negatively stained specimen was prepared 30 min after the start of assembly. The protofilaments of these microtubules are readily apparent. (B) Polymerization at physiological temperature. To initiate assembly at 0 °C, GTP (final concentration 1 mM) was added to a solution of *N. gibberifrons* tubulin (4.4 mg/mL in PME buffer) lacking free nucleotide. After 70 min, a sample was prepared for negative-stain electron microscopy. The bar in each panel represents 100 nm.

between 0 and 24 °C, and addition of podophyllotoxin (final concentration 5×10^{-5} M) to solutions of the piscine microtubules produced rapid depolymerization (data not shown).

Temperature Dependence of the Critical Concentration for Microtubule Assembly. At a given temperature, the formation

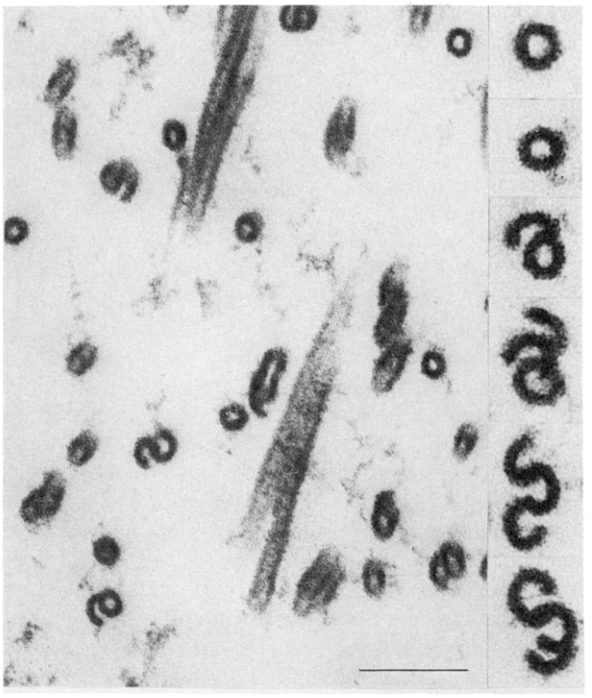

FIGURE 4: Thin-section electron microscopy of microtubule polymer polymerized from tubulin of the icefish *C. aceratus*. A solution of icefish tubulin (2.6 mg/mL in PME buffer containing 1 mM GTP) was warmed from 0 to 18 °C at zero time. Thirty minutes after the start of assembly, a sample was fixed by addition of glutaraldehyde/tannic acid and then prepared for thin-section electron microscopy as described under Experimental Procedures. The bar represents 100 nm. The sidebar shows cross sections of a nonrepresentative sample of microtubules and several polymorphic structures, including "hooked" microtubules and serpentine sheets, at approximately 2× greater magnification.

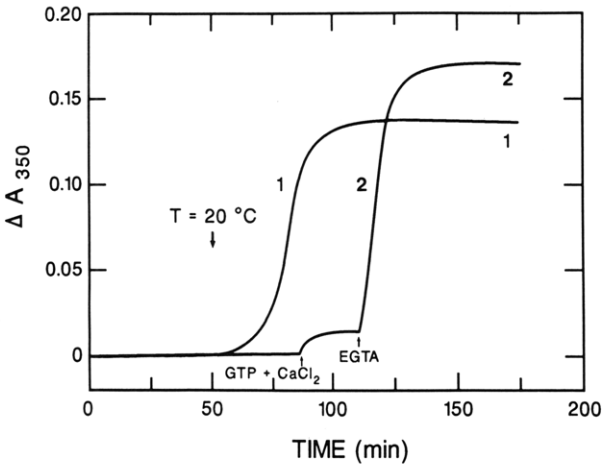

FIGURE 5: Effects of GTP and calcium ion on the polymerization of tubulin from *N. gibberifrons*. Two samples (1 and 2) of purified tubulin (0.65 mg/mL) were prepared at 0 °C in a nucleotide-free buffer system (0.1 M Pipes–NaOH, 2 mM $MgSO_4$, 1 mM EGTA, and 1 mM DTE, pH 6.82). GTP was added to sample 1 to a final concentration of 1 mM at zero time, the two samples were warmed from 0 to 20 °C at t = 50 min, and polymerization was monitored as the change in apparent absorbance at 350 nm (ΔA_{350}). $CaCl_2$, GTP, and EGTA (final concentrations of 10, 1, and 12 mM, respectively) were added to sample 2 at the times indicated by the arrows.

of microtubules in vitro requires a minimal, or "critical", concentration of tubulin dimers. We measured the critical concentrations for polymerization of tubulins from two Antarctic fishes at eight temperatures between 0 and 18 °C by means of the quantitative sedimentation assay of Johnson and Borisy (1975). Figure 6A shows determinations of the critical concentrations of *N. gibberifrons* tubulin at four temperatures.

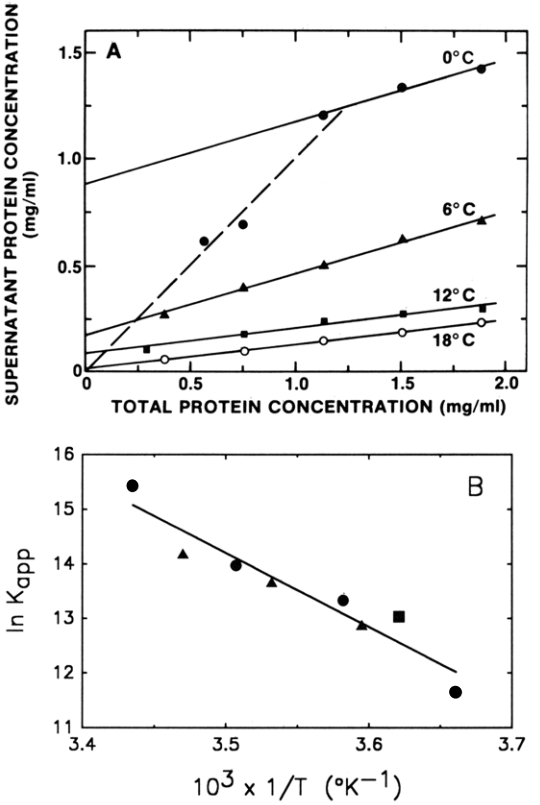

FIGURE 6: Thermodynamics of the polymerization of Antarctic fish microtubules in vitro. (A) Temperature dependence of the critical concentration. Critical concentrations for the polymerization of *N. gibberifrons* tubulin were measured at temperatures between 0 and 18 °C by means of a quantitative sedimentation assay (see Experimental Procedures). Concentrations of the supernatant (monomer) fractions are plotted as a function of the total tubulin concentration for determinations at 0 °C (closed circles), at 6 °C (triangles), at 12 °C (squares), and at 18 °C (open circles). For each temperature, the critical concentration was estimated as the y intercept of the linear regression line through the corresponding monomer data points (neglecting data points obtained at total protein concentrations that did not yield detectable polymer pellets). (B) van't Hoff analysis of the apparent equilibrium constant for microtubule elongation. The natural logarithm of the apparent equilibrium constant for microtubule elongation (i.e., the natural logarithm of the reciprocal of the critical concentration) is shown as a function of the reciprocal of the absolute temperature. Three tubulin preparations from two Antarctic fishes were used in these studies: (circles) *N. gibberifrons* tubulin, preparation 1; (triangles) *N. gibberifrons* tubulin, preparation 2; (square) *N. coriiceps neglecta* tubulin. The best-fitting straight line through the data points was determined by linear regression analysis.

Values for the critical concentrations of the Antarctic fish tubulins, estimated as the y intercepts of the linear regression lines through the appropriate supernatant (monomer) data points (see Experimental Procedures), ranged from 0.87 mg/mL at 0 °C to 0.02 mg/mL at 18 °C. Thus, tubulins from Antarctic fishes form microtubules in vitro at physiological temperatures and at low protein concentrations.

The positive slopes of the monomer lines (Figure 6A) indicate that some of the tubulin did not participate in the assembly reactions. The proportion of this "inactive" tubulin increased from 12% at 18 °C to 29% at 0 °C. The latter observation suggests that there may be subspecies of the piscine tubulin that differ in their capacities to polymerize at low temperatures. To address this hypothesis, samples of *N. gibberifrons* tubulin were polymerized to steady state at several temperatures (0, 8, and 16 °C) by the assembly protocol used for measurement of critical concentrations (see Experimental Procedures), the polymer and monomer fractions were separated by centrifugation, and the isotype compositions of the

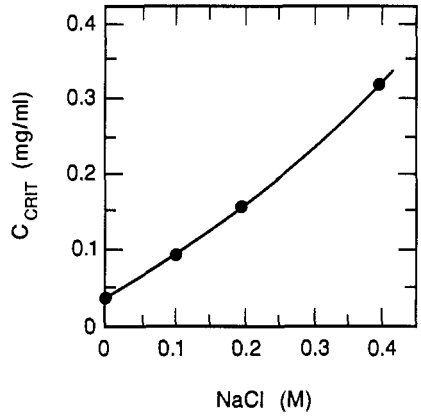

FIGURE 7: Salt dependence of the critical concentration for polymerization of *N. gibberifrons* tubulin. Critical concentrations (C_{CRIT}) were measured at 10 °C in a buffer (PMG plus 1 mM DTT, 8 mM $CaCl_2$, and 10 mM EGTA) supplemented with concentrations of NaCl from 0 to 0.4 M (see Experimental Procedures). The line is drawn to indicate the trend in the data.

fractions were examined by high-resolution isoelectric focusing. Microtubules collected at low and high temperatures contained similar, if not identical, distributions of tubulin variants, as did the corresponding monomer fractions (data not shown). Apparently, the tubulin "inactive" in microtubule assembly at low temperatures does not constitute a poorly polymerizing subset of the total tubulin pool.

Figure 6B presents the critical concentration data in the form of a van't Hoff plot. For this analysis, the apparent equilibrium constant for microtubule elongation (i.e., the addition of a tubulin dimer to the end of a microtubule) was assumed to equal the reciprocal of the critical concentration (Oosawa & Higashi, 1967; Gaskin et al., 1974; Lee & Timasheff, 1977). The data points, obtained for three tubulin preparations from two fish species, appear to be adequately fit by a straight line ($r = -0.956$). The standard enthalpy change, $\Delta H°$, for elongation is +26.9 kcal/mol, and the corresponding standard entropy change, $\Delta S°$, is +123 eu. Clearly, the polymerization of microtubules from Antarctic fish tubulins is entropically driven.

Salt Dependence of Microtubule Assembly. Thermochemically, hydrophobic interactions and electrostatic (ionic) bonds make positive contributions to the entropy of subunit association, whereas hydrogen bonds and van der Waals contacts produce negative entropy changes (Cantor & Schimmel, 1980; Ross & Subramanian, 1981). Thus, the results shown in Figure 6B indicate that the energetics of the polymerization of Antarctic fish microtubules are dominated by hydrophobic interactions and/or ionic bonds. To evaluate the relative contributions of these two interactions, we measured the critical concentration for assembly of *N. gibberifrons* tubulin at 10 °C as a function of the concentration of NaCl added to the buffer.[2] As shown in Figure 7, the critical concentration increased monotonically from 0.041 to 0.34 mg/mL as the salt concentration rose from 0 to 0.4 M. This modest increase of the critical concentration in response to added NaCl, corresponding to a reduction of 1.2 kcal/mol in the free energy of elongation, indicates that hydrophobic interactions make major contributions to the entropic control of microtubule formation by the tubulins of Antarctic fishes. It is clear that the tubulins of these cold-adapted fishes polymerize efficiently at NaCl concentrations that suppress the assembly of tubulins (Himes et al., 1977) or total microtubule proteins (Olmsted & Borisy, 1975) from mammals.

Discussion

The work presented here demonstrates that pure tubulins from Antarctic fishes (Figure 1) polymerize efficiently to yield microtubules and microtubule polymorphs (Figures 3 and 4) at temperatures between 0 and 20 °C (Figure 6B). The polymers formed are dynamic, not metastable, structures; they polymerize and depolymerize reversibly in response to changes in temperature (Figures 2 and 5) or in calcium ion concentration (Figure 5). MAPs or dissociable, cold-stabilizing ligands are not required for polymerization of the fish tubulins at low temperatures. Rather, the assembly and cold stability of the microtubules of Antarctic fishes must reflect, in large part, adaptive changes in the tubulin subunits themselves. It appears likely that some of the modifications alter the domains for interdimer contact, thus yielding tubulins with association constants appropriate for polymerization at the habitat temperatures of these fishes.

Thermodynamics of Microtubule Assembly by the Tubulins of Cold-Adapted Fishes. Microtubule assembly in vitro displays the characteristics of a nucleated condensation polymerization reaction (Johnson & Borisy, 1977). Oosawa and Higashi (1967) have shown that the equilibrium constant for addition of monomers to the ends of helical polymers (e.g., microtubules, actin filaments) is approximately equal to the reciprocal of the critical monomer concentration. Because GTP is hydrolyzed irreversibly during and following polymerization, solutions of microtubules approach a steady state and do not strictly attain equilibrium (Correia & Williams, 1983). However, the near-identity of critical concentrations for microtubule assembly supported by GTP and by nonhydrolyzable analogues of GTP [e.g., guanosine 5′-(β,γ-methylenetriphosphate) and guanosine 5′-(β,γ-imidotriphosphate) (Karr et al., 1979)] indicates that the hydrolysis of GTP is not coupled thermodynamically to the *elongation* reaction of microtubules. Rather, dephosphorylation of GTP appears to be necessary to sustain steady-state reactions such as subunit treadmilling (Terry & Purich, 1980; Cote & Borisy, 1981; Margolis, 1981) or dynamic instability (Mitchison & Kirschner, 1984; Carlier et al., 1987; O'Brien et al., 1987). Nonetheless, application of the theory of Oosawa and Higashi (1967) to microtubules at steady state should be a valid method for analysis of the energetics of microtubule elongation.

The large, positive values for $\Delta H°$ (+26.9 kcal/mol) and for $\Delta S°$ (+123 eu), determined by van't Hoff analysis of the apparent equilibrium constants for microtubule elongation, indicate that the polymerization of tubulins from two Antarctic fishes, *N. gibberifrons* and *N. coriiceps neglecta*, is entropically driven. Similar results were obtained for tubulin purified from the Antarctic fish *P. borchgrevinki* by cycles of temperature-dependent microtubule assembly and disassembly (Williams et al., 1985), but these investigators reported smaller values for $\Delta H°$ and $\Delta S°$ (+13.7 kcal/mol and +74 eu, respectively). The discrepancy between the two data sets probably results from methodological differences rather than from the species of fish studied. Three differences may be particularly significant. First, the tubulins used in this study were subjected to temperatures no higher than 24 °C during purification and subsequent experimentation, whereas the tubulins examined by Williams et al. (1985) were exposed to temperatures of 37 °C when prepared by the cycling protocol. Perhaps metastable conformational changes attributable to the different thermal histories of the two preparations have

[2] High concentrations of antichaotropic, or structure-making, salts, such as NaCl, strengthen hydrophobic interactions and weaken electrostatic bonds (Shansky et al., 1989).

altered the polymerization behavior of the tubulins. Second, we employed a quantitative sedimentation assay and multiple protein concentrations to obtain estimates of the critical concentrations that were corrected for inactive tubulin (Figure 6A). Williams et al. (1985), in contrast, used turbidimetry or sedimentation to measure critical concentrations at single protein concentrations; thus, their values represent the sum of active tubulin, inactive tubulin, and nonmicrotubule protein (if any). Third, the buffer system employed for our critical concentration measurements (see legend to Figure 6) differed slightly in composition from that (0.1 M Pipes–KOH, 1 mM EGTA, 2 mM $MgSO_4$, 2 mM DTE, and 1 mM GTP, pH 6.9) used by Williams et al. (1985). Despite these differences, both studies agree that the polymerization of Antarctic fish tubulins is under entropic control. However, the entropic contribution to the polymerization energetics appears to be substantially greater than prior estimates (Williams et al., 1985) indicated.

Recently, Strömberg et al. (1986) described the isolation and characterization of microtubule proteins from brain tissue of the cold-water Atlantic cod *Gadus morhua*. Microtubules obtained by one cycle of temperature-dependent polymerization and depolymerization were cold-labile, and the critical concentration for microtubule assembly at 30 °C was large (0.8 mg/mL). Clearly, the polymerization behavior of the cod microtubule proteins differs from that of the tubulins of Antarctic fishes. The central nervous system of the gadid fish presumably does contain microtubules that are stable at physiological temperatures near 0 °C. If so, the cold lability of the purified cod microtubules may reflect loss of cold-stable microtubules or of a cold-stabilizing factor during preparation. Given the low yield of microtubule protein obtained (0.07 mg/g of brain tissue), the first explanation appears most plausible. Further studies will be necessary to determine whether the mechanisms that confer cold-stability on the microtubules of cold-adapted fishes from the austral and boreal oceans are similar or different.

Polymerization Thermodynamics of Other Tubulins. The values of $\Delta H°$ and $\Delta S°$ of the Antarctic fish tubulins are significantly greater than those found for other pure tubulins. Robinson and Engelborghs (1982) reported that the van't Hoff plot for the polymerization of porcine brain tubulin in a buffer system (50 mM Mes, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, 70 mM KCl, and 1 mM NaN_3, pH 6.4) containing 8% dimethyl sulfoxide was linear over the temperature range 10–35 °C; the standard enthalpy change was +6.3 kcal/mol, and the standard entropy change was +44.4 eu. By contrast, Lee and Timasheff (1977), working with bovine tubulin in a phosphate buffer system containing 3.4 M glycerol and 16 mM $MgCl_2$, described a curved van't Hoff plot that reflected a negative heat capacity change. As temperature increased from 23 to 37 °C, values for $\Delta H°$ decreased from +22.8 to +2.2 kcal/mol, while values of $\Delta S°$ decreased from +98 to +30 eu (Lee & Timasheff, 1977). Finally, polymerization of egg tubulin from the sea urchin *S. purpuratus* (physiological temperature range 15–18 °C), in buffer systems similar to that used in this study, is characterized by standard enthalpy and entropy changes of ~11–16 kcal/mol and ~61–79 eu, respectively.[3] While the comparisons are limited and the buffer conditions employed in these studies are not identical, it appears that the entropic control of tubulin polymerization increases with decreasing average body temperature.

[3] Estimates of the standard enthalpy and standard entropy changes for polymerization of *S. purpuratus* egg tubulin were derived from the critical concentration data of Suprenant and Rebhun (1983), Detrich and Wilson (1983), and Detrich et al. (1985a) by van't Hoff analysis.

Molecular Interactions in Antarctic Fish Microtubules. The ability to form microtubules efficiently at different body temperatures may involve changes in the bond types that stabilize the polymer. With respect to other tubulins, the large, positive values for the enthalpy and entropy of polymerization of the Antarctic fish tubulins suggest that the piscine tubulins rely to a greater extent on entropy-driven molecular interactions at their domains of dimer–dimer contact in the microtubule. Furthermore, the minimal perturbation of the critical concentration (hence, the equilibrium constant for elongation) by high ionic strength (Figure 7) indicates that the greater entropic control results from an increased dependence on hydrophobic interactions (relative to ionic bonds). Together, these results suggest that the Antarctic fishes have overcome the destabilizing influence of low temperatures on entropically driven reactions through the evolution of tubulin dimers that form increased numbers of (or qualitatively stronger) hydrophobic interactions at their interdimer contact surfaces.

Thermodynamics of Actin Polymerization. Recently, Swezey and Somero (1982) examined the polymerization energetics of skeletal muscle actins from 14 vertebrate species having average body temperatures from −1.9 to +39 °C. In contrast to the trend observed for pure tubulins (see above), they found that the $\Delta H°$ and $\Delta S°$ of actin polymerization *increased* in a regular fashion with increasing average body temperature. The polymerization of actins from Antarctic and deep-sea fishes, the organisms with the lowest body temperatures, was accompanied by the smallest values for $\Delta H°$ and $\Delta S°$. Swezey and Somero (1982) suggested that cold-adapted organisms (e.g., Antarctic and deep-sea teleosts) are less dependent on hydrophobic interactions for the maintenance of their actin filaments than are warm-blooded organisms. Thus, there appear to be at least two adaptive mechanisms that enable cytoskeletal polymers to be assembled efficiently at low temperatures: (1) quantitative and/or qualitative modification of the bond types at subunit contact surfaces to provide an entropy of association sufficient to overcome unfavorable enthalpy changes (e.g, tubulin); or (2) mitigation of destabilizing enthalpy changes by greater reliance on bond types that make negative contributions to the overall enthalpy of polymerization (e.g., actin).

Comparison of Cold-Stable Microtubules from Homeotherms and from Poikilotherms. In addition to their cold-labile microtubules, many mammalian cells contain a subset of "cold-stable" microtubules that resist depolymerization at low temperatures (0–4 °C) (Jones et al., 1980). For example, during metaphase, the interpolar microtubules in the spindles of mitotic PtK_1 cells depolymerize rapidly at low temperatures, whereas the kinetochore microtubules are stable (Brinkley & Cartwright, 1975). Thus, it is appropriate to ask whether the mechanisms that generate cold-stable microtubules in homeotherms and in cold-living poikilotherms are similar or different. The available evidence indicates that the latter conclusion is correct.

Webb and Wilson (1980) reported that purified cold-stable microtubules from mouse brain were composed of tubulin that was indistinguishable in subunit composition from the tubulin of cold-labile microtubules. The stability of these microtubules at low temperatures appeared to be conferred by a low molecular weight factor whose association with microtubules was controlled by calcium ion. Subsequently, Margolis and coworkers (Job et al., 1983; Pirollet et al., 1983; Margolis et al., 1986) have shown that substoichiometric incorporation of one or a few non-tubulin proteins, termed STOPs ("stable tubule only polypeptides"), into microtubules renders a portion of the

polymer population cold-stable. However, de novo polymerization of mammalian cold-stable microtubules from their constituent proteins requires the same elevated temperatures that promote the assembly of cold-labile microtubules (Webb & Wilson, 1980; Job et al., 1983; Pirollet et al., 1983; Margolis et al., 1986). Thus, in contrast to the microtubules of Antarctic fishes, these mammalian cold-stable microtubules must be considered metastable polymers whose stability is not intrinsic to their tubulin subunits, but rather is mediated by non-tubulin factors. The physiological role of mammalian cold-stabilizing factors may be to modulate microtubule dynamics in the neuronal axon (Margolis et al., 1986) or in the mitotic spindle (Job et al., 1982).

The cold-stable microtubules of Webb and Wilson (1980) and of Margolis and his colleagues (Job et al., 1982, 1983; Pirollet et al., 1983; Margolis et al., 1986) were purified from a *cold-soluble* supernatant fraction of mammalian brain tissue; such preparations may well be depleted of endogenous cold-stable polymers of tubulin (lost to the pellet following homogenization of brain tissue and subsequent centrifugation at low temperature). Recently, Brady et al. (1984) and Binet and Meininger (1988) examined the tubulins contained in calcium- and cold-stable particulate fractions from mammalian neural tissues. They attributed the cold insolubility of the particulate tubulins to specific tubulin variants and suggested that these modified tubulins are able to form cold-stable microtubules in vivo. However, this latter conclusion must be regarded as tentative because morphological evidence demonstrating that the particulate fractions actually contain bona fide cold-stable microtubules or that the particulate tubulins can form cold-stable microtubules in vitro (as do the tubulins of Antarctic fishes) has not been presented. Furthermore, the particulate tubulins of mammals resist extraction (Brady et al., 1984; Binet & Meininger, 1988) under conditions (see above) that readily solubilize the tubulins of Antarctic fishes. Thus, it appears unlikely that the tubulins of Antarctic fishes share structural or functional properties with the cold-insoluble tubulins of mammals.

Summary: *Conservation of the Critical Concentration for Microtubule Assembly*. The interspecific differences in the polymerization thermodynamics of tubulins suggest strongly that evolutionary changes have occurred at sites of interdimer contact. These modifications have produced tubulins that possess, at physiologically appropriate temperatures, comparable critical concentrations for microtubule assembly. For example, critical concentrations for polymerization of tubulins from Antarctic fishes, from the sea urchin, and from mammals, when measured in vitro in buffers of similar composition and *at the physiological body temperatures* of these organisms, fall in the range 0.71–2.5 mg/mL (Herzog & Weber, 1977; Suprenant & Rebhun, 1983; Detrich et al., 1985a; Williams et al., 1985). As a consequence of this interspecific conservation of the critical concentration, each of these organisms is able to assemble and disassemble microtubules efficiently at its normal body temperature.

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CORRECTIONS

Direct Observation of the Enzyme–Intermediate Complex of 5-Enolpyruvylshikimate-3-phosphate Synthase by ^{13}C NMR Spectroscopy, by Paul N. Barlow, Richard J. Appleyard, Ben J. O. Wilson, and Jeremy N. S. Evans*, Volume 28, Number 20, October 3, 1989, pages 7985–7991.

Page 7989. In Table I, the assignment for the chemical shift at 152.4 ppm should read C-2 enzyme-free PEP.

Plastocyanin Cytochrome *f* Interaction, by Larry Z. Morand, Melinda K. Frame, Kim K. Colvert, Dale A. Johnson, David W. Krogmann, and Danny J. Davis*, Volume 28, Number 20, October 3, 1989, pages 8039–8047.

Page 8043. Figure 4 should be as follows:

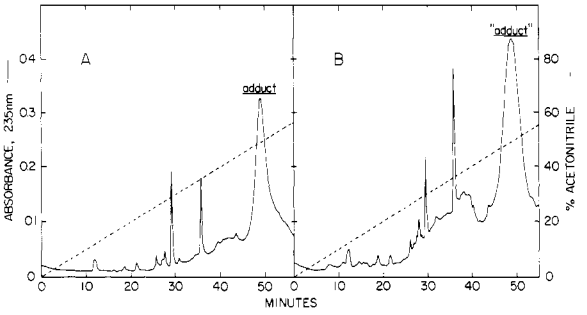

FIGURE 4: HPLC purification of PC–cyt *f* adduct before and after CNBr digestion. Prior to sequencing experiments, the PC–cyt *f* adduct (panel A) and the CNBr digest of the PC–cyt *f* adduct (panel B) were purified by HPLC. CNBr digestion and HPLC procedures were as described under Materials and Methods.

Page 8043. The display at the bottom of Table V should appear as follows:

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                          1       7
PC N-terminus             V E V L L G G -

cyt f N-terminus          Y P I F A Q Q -


                             58      62
PC (res 58-62)               S E x D L
                              ¦
                             (E)
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