Polymerization of Antarctic Fish Tubulins at Low Temperatures: Role of Carboxy-Terminal Domains†

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ABSTRACT: We have proposed previously that the efficient polymerization of tubulins from Antarctic fishes at low, physiological temperatures (−1.8 to +2 °C) may result in part from adaptations (e.g., reductions in acidic residues) located in their carboxy-terminal (C-terminal) tails [Detrich & Overton (1986) *J. Biol. Chem. 261*, 10922−10930]. To test this hypothesis, we have examined the polymerization of Antarctic fish neural tubulins modified at their C termini by proteolysis or by neutralization of carboxyl groups. Addition of subtilisin to low concentrations of *Notothenia coriiceps* tubulin induced a biphasic assembly reaction: stage I corresponded to the C-terminal cleavage of β chains to produce $\alpha\beta_s$ dimers, and stage II coincided with the slower, C-terminal cleavage of α chains to yield $\alpha_s\beta_s$. Both stage I and stage II polymers consisted of protofilament sheets and microtubules with attached sheets. The critical concentration for assembly of the stage II polymer was at least 10-fold lower than that of untreated tubulin. Neutralization of Glu and Asp carboxyls in *Gobionotothen gibberifrons* microtubules by the carbodiimide-catalyzed incorporation of glycine ethyl ester (GEE) moieties produced a tubulin, modified largely in its C termini, that assembled more readily than did control tubulin. When 12 GEE groups were incorporated per dimer, the critical concentrations for assembly of modified tubulin at 5−10 °C were 2−3-fold smaller than those for the unmodified protein. Comparably modified bovine tubulin (10 GEE/dimer) assembled at 37 °C with a critical concentration 2.6-fold lower than that for the unmodified tubulin. GEE-modified *G. gibberifrons* tubulin, like modified bovine tubulin, produced microtubules which were indistinguishable from those formed by control tubulin. Our results suggest that the C termini of Antarctic fish tubulin are not major loci for cold adaptation of microtubule assembly.

Subjected to an increasingly severe thermal environment as the Southern Ocean cooled, the coastal fishes of the Antarctic diverged from temperate fishes approximately 25−40 million years ago [DeWitt, 1971; reviewed by Eastman (1993)] and evolved cellular and biochemical adaptations that preserve macromolecular structure and function at their now chronically low body temperatures (−1.86 to +2 °C). Their cytoplasmic microtubules, in contrast to those of homeotherms (e.g., mammals), must assemble from their component proteins [tubulin $\alpha\beta$ dimers and microtubule-associated proteins (MAPs)[1]] and perform their functions at these cold temperatures.[2] Pure brain tubulins from several Antarctic fish species form microtubules efficiently at temperatures as low as −1.8 °C in the absence of MAPs (Williams et al., 1985; Detrich et al., 1989). Polymerization is strongly entropy driven, which probably reflects an increased reliance, relative to homeothermic tubulins, on hydrophobic interactions at sites of interdimer contact. Furthermore, these microtubules are dynamic polymers at low temperatures, but subunit exchange via dynamic instability or treadmilling is slow (Himes & Detrich, 1989; Billger et al., 1994). Brain MAPs from Antarctic fishes make additional contributions to the energetics of microtubule formation, but they possess no greater assembly-promoting activity at low temperatures than do mammalian MAPs (Detrich et al., 1990). Together, our results suggest that the cold-adapted phenotype of microtubule assembly in Antarctic fishes is based largely, if not exclusively, on molecular evolution of the α- and β-tubulin subunits.

Our long-term objective is to determine the molecular adaptations of tubulins from the cold-adapted Antarctic fishes. We have shown previously that the brain tubulin isoforms of Antarctic fishes are more basic than are those of a temperate fish and a mammal, due most likely to a reduced content of glutamyl residues (Detrich & Overton, 1986, 1988). Furthermore, the C-terminal domains of these tubulins differ from those of mammalian tubulins in their sensitivity to cleavage by subtilisin, a protease that cleaves

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[1] Abbreviations: C_c, critical concentration; DEAE, (diethylamino)-ethyl; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GEE, glycine ethyl ester; GTP, guanosine 5′-triphosphate; MAP, microtubule-associated protein; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

[2] Although small quantities of cold-stable microtubules are present in some mammalian tissues (e.g., brain), they constitute metastable subpopulations whose disassembly is prevented by associated proteins (Job et al., 1982; Margolis et al., 1986). When depolymerized by nonthermal methods, these microtubules reassemble only at elevated temperatures (30−37 °C).

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small, acidic peptides from the C termini of the tubulin chains (Detrich et al., 1987, 1992). These results suggest that the charge status of the Antarctic fish tubulins or the structure of their C termini, or both, may contribute to cold adaptation of microtubule assembly.

The assembly of microtubules is controlled in part by the highly acidic carboxy-terminal tails of their α- and β-tubulin subunits. The C-terminal regions of most tubulins are rich in glutamyl and aspartyl residues (Sullivan, 1988), are highly charged at physiological pH, exist in an extended, accessible conformation (Ringel & Sternlicht, 1984) in the dimer (Sackett & Wolff, 1986) and in the microtubule (Breitling & Little, 1986), and contain binding sites for MAPs (Serrano et al., 1984a, 1985; Littauer et al., 1986; Maccioni et al., 1988; Vera et al., 1988). Release of small, C-terminal fragments (2−4 kDa), first from β tubulin and then from the α chain of the native dimer by treatment with subtilisin, reduces by 10−50-fold the critical concentration (Serrano et al., 1984b; Bhattacharyya et al., 1985; Sackett et al., 1985) for polymer formation [hooked microtubules and protofilament sheets (Serrano et al., 1984b, 1988; Sackett et al., 1985; White et al., 1987)] by the cleaved protein. Thus, the intact C termini of the tubulin subunits may hinder polymerization by generating electrostatic repulsion as dimers approach the end of an elongating microtubule. MAP 2 and tau, through their cationic, C-terminal domains, interact with the acidic C termini of the tubulins (Lee et al., 1988; Lewis et al., 1988; Himmler et al., 1989; Noble et al., 1989; Vera et al., 1989; Cleveland, 1990; Vallee, 1990), thereby neutralizing the negative charges and relieving the inhibition of assembly (Serrano et al., 1984b; Bhattacharyya et al., 1985). Similarly, conversion of the carboxyl side chains of C-terminal Asp and Glu residues to amides decreases the critical protein concentration for assembly (Mejillano & Himes, 1991) and stabilizes microtubules against depolymerization (Mejillano et al., 1992). On the basis of these considerations, we have proposed that the efficient polymerization of Antarctic fish tubulins at low, physiological temperatures (−1.8 to +2 °C) may result in part from adaptations that reduce the content of acidic residues (contributed by the primary sequence and/or by posttranslational polyglutamylation) in their C-terminal tails (Detrich & Overton, 1986; Detrich et al., 1992; Detrich & Parker, 1993). To test this hypothesis, which predicts reduced responsiveness of Antarctic fish tubulins to C-terminal removal or neutralization, we have examined the polymerization properties of brain tubulins from two Antarctic rockcods [*Notothenia coriiceps* and *Gobionotothen* (*Notothenia*) *gibberifrons*] following C-terminal cleavage by subtilisin or carbodiimide-mediated amidation of carboxyl side chains. We find that these treatments reduce by comparable extents the critical concentrations for polymerization of fish or bovine tubulins at their respective physiological temperatures. We conclude that the C termini of brain tubulins do not play a major role in cold adaptation of microtubule assembly. A preliminary report of this work has appeared (Singer et al., 1991).

EXPERIMENTAL PROCEDURES

Materials. Pipes, EGTA, GEE hydrochloride, GTP (type II-S), PMSF, SDS, and subtilisin BPN′ were obtained from Sigma. [^{14}C]GEE hydrochloride was purchased from New England Nuclear, EDC from Pierce Chemical Co., and DEAE-Sephacel from Pharmacia LKB Biotechnologies Inc. Acrylamide and *N*,*N*′-methylenebis(acrylamide) were products of Bio-Rad Laboratories. Spurr's resin was obtained from Ernest Fullam, Inc., and glutaraldehyde, OsO_4, and uranyl acetate were supplied by Ted Pella, Inc. Other chemicals were reagent grade.

Collection of Fishes. Specimens of two Antarctic rockcods, *N. coriiceps* and *G. gibberifrons*, were collected by bottom trawling from the *R/V Polar Duke* near Low and Brabant Islands in the Palmer Archipelago. They were transported alive to Palmer Station, Antarctica, and maintained in seawater aquaria at −1 to +1 °C.

Purification of Tubulin. The purification of MAP-free tubulin from brains of the Antarctic fishes has been described previously (Detrich & Overton, 1986; Detrich et al., 1989; Himes & Detrich, 1989). The procedure involves DEAE-Sephacel chromatography of a high speed supernatant of brain homogenate, followed by polymerization and recovery of the centrifuged microtubules. Microtubule protein from bovine brain was isolated by three cycles of microtubule assembly and disassembly (Shelanski et al., 1973). Tubulin was separated from MAPs by polymerization in a high ionic strength buffer containing 10% (v/v) DMSO followed by chromatography on a phosphocellulose/Bio-Gel P-10 piggyback column (Algaier & Himes, 1988).

Digestion of Antarctic Fish Tubulin with Subtilisin. Brain tubulin (0.7−2 mg/mL) from *N. coriiceps* was incubated with 1% or 2% (w/w) subtilisin at 5 or 10 °C in MME buffer (0.1 M Mes−NaOH, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.7 at 20 °C) containing 1 mM GTP, and polymerization was followed by turbidimetry (apparent ΔA_{350}). In some experiments, aliquots were withdrawn at intervals, proteolysis was terminated by addition of PMSF to a final concentration of 1 mM, and the samples were placed on ice. Intact and subtilisin-cleaved tubulins were analyzed by SDS−polyacrylamide gel electrophoresis, and polymerization products were examined by electron microscopy as described below.

Preparation of Chemically Modified Tubulins. Carboxyl group modification of Antarctic fish tubulin was performed by a modification of the carbodiimide-mediated amidation protocol of Mejillano and Himes (1991). Pellets of brain microtubules from *G. gibberifrons* were resuspended in PME buffer (0.1 M Pipes−NaOH, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.9 at 20 °C) by use of a Dounce homogenizer. After incubation at 0 °C for 15 min, the suspension was clarified by centrifugation (40000*g*, 20 min, 0 °C), and the tubulin-containing supernatant was recovered and adjusted to a concentration of 1 mg/mL. To initiate polymerization, GTP was added to the tubulin solution to a final concentration of 1 mM, and the solution was warmed to 20 °C for 15 min. [^{14}C]Glycine ethyl ester ([^{14}C]GEE; 2×10^4 cpm/μmol) and the carbodiimide EDC were then added sequentially to the microtubule solution to final concentrations of 0.1 M (or 0.3 M) and 3 mM, respectively. The polymer was incubated with the modifying agents at 20 °C for periods up to 120 min. Aliquots (300 μL) of the solution were layered over 2-mL cushions of PME containing 40% sucrose, and modified polymer was collected as pellets by centrifugation (165000*g*, 60 min, 20 °C). The pellets were rinsed five times with PME buffer at room temperature and resuspended in ice-cold PME by Dounce homogenization, and the resulting solution was incubated at 0 °C for 15 min. Finally, cold-insoluble debris was removed by centrifugation (40000*g*, 20

min, 0 °C), and the supernatant, containing GEE-modified tubulin, was recovered. Protein concentrations of the supernatants were measured (see below), and [^{14}C]GEE incorporation was determined by scintillation counting. Bovine brain tubulin was modified in a similar fashion. Tubulin (3 mg/mL) was incubated in PME buffer containing 10% DMSO and 1 mM GTP at 37 °C for 20 min. [^{14}C]-GEE (0.1 M) and EDC (0.5 mM) were then added, and the incubation was continued for 60 min. The temperature of the sucrose centrifugation step was also increased from 20 to 37 °C.

Determination of Critical Concentrations. Critical concentrations (C_c) for the polymerization of unmodified and modified Antarctic fish tubulin at 5 and 10 °C, and for bovine brain tubulin at 37 °C, were determined by a modification of the sedimentation assay of Johnson and Borisy (1975). Different concentrations of tubulin containing 1 mM GTP were assembled in cuvettes, and development of turbidity was monitored by use of Beckman DU64 or Shimadzu UV2100U spectrophotometers, both of which were equipped with temperature-controlled multicuvette chambers. After the apparent absorbance of the assemblies had reached a steady-state plateau, 0.2 mL of each sample was centrifuged at the same temperature as the assembly reaction for 20 min at 40000*g*. The supernatants were carefully removed and their protein concentrations were determined. The critical concentration at each temperature was obtained from the *y*-intercepts of extrapolated linear regression lines of plots of supernatant protein concentration *vs* total protein concentration.

Limited Proteolysis of Modified Tubulin. Limited proteolysis was used to determine whether the modified carboxyl groups were located primarily in the C-terminal regions of tubulin. Subtilisin [4% (w/w)] was added for 30 min at 25 °C to a sample of 1 mg/mL *G. gibberifrons* tubulin that had been polymerized in the presence of 1 mM GTP and 10% DMSO for 15 min. The protease action was stopped by the addition of 2 mM PMSF in DMSO, and the assembled material was collected by centrifugation at 200000*g* for 4 min. The pellet was resuspended in PME buffer, and the digestion products were analyzed by SDS−polyacrylamide gel electrophoresis.

Electrophoresis. SDS−polyacrylamide gel electrophoresis (Laemmli, 1970) was performed on slab gels [8% or 9% (w/v), pH 9.2] modified as described by Bhattacharyya et al. (1985). To quantitate the incorporation of [^{14}C]GEE in the α and β subunits, and in the proteolytic fragments, the stained and destained gels were washed with water for 30 min, bands were excised and dissolved in 30% H_2O_2 for 12 h at 80 °C, and scintillation fluid was added prior to counting.

Electron Microscopy. For negative staining, samples from an assembly reaction were diluted 10-fold into PME or MME buffer containing 1% (w/v) glutaraldehyde. After 1 min, the fixed material was placed on Formvar- and carbon-coated copper grids, washed three times with H_2O, and stained with 2% (w/v) uranyl acetate. For thin-sectioning, polymer-containing samples were incubated overnight (approximately 12 h) at 4 °C with a 3-fold volume excess of 3% glutaraldehyde and 4% tannic acid in 50 mM phosphate buffer, pH 6.8. The fixed material was collected by centrifugation at 40000*g* for 20 min, postfixed in 1% OsO_4, dehydrated with acetone, and embedded in Spurr's resin. Sections were collected on uncoated copper grids and stained with 2% (w/v) uranyl acetate and then 0.3% (w/v) lead citrate. Grids were viewed with a JEOL EXII transmission electron microscope.

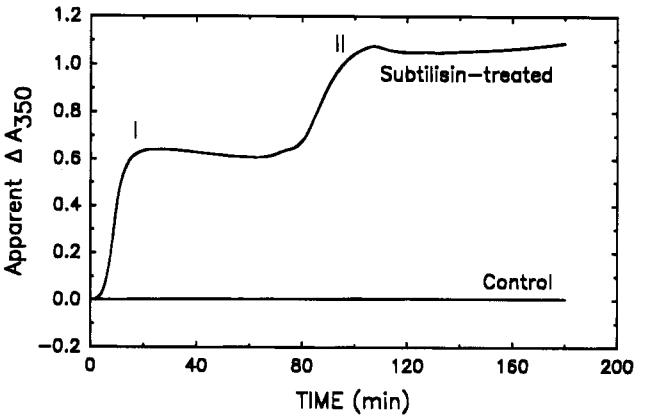

FIGURE 1: Subtilisin-induced polymerization of Antarctic fish tubulin. Brain tubulin (2.0 mg/mL) from *N. coriiceps* was incubated with 2% (w/w) subtilisin in MME buffer and 1 mM GTP at 5 °C, and polymerization was monitored by turbidimetry (apparent ΔA_{350}). The control sample was prepared and treated identically, except for the omission of subtilisin.

Protein Determinations. Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin as the standard.

RESULTS

Subtilisin-Induced Polymerization of Antarctic Fish Tubulin. Figure 1 shows the subtilisin-induced polymerization of *N. coriiceps* brain tubulin at 5 °C. Following the addition of subtilisin, two distinct stages of turbidity development (i.e., assembly), labeled I and II, were observed. Surprisingly, the stage I polymer formed by *N. coriiceps* tubulin, when examined by electron microscopy, consisted largely of protofilament sheets and small amounts of "hooked" microtubules (i.e., microtubules with sheets attached to their walls) (Figure 2A) rather than the bidirectionally hooked microtubules (Serrano et al., 1988) or ring polymers (Bhattacharyya et al., 1985) produced by stage I mammalian brain tubulins. Stage II polymer (Figure 2B,C) resembled the stage I polymorphs, but the proportion of multiply hooked microtubules was much greater. At 5 and 10 °C, the critical concentrations for assembly of the piscine stage II polymer, like those for subtilisin-cleaved mammalian brain tubulins at 35−37 °C (Serrano et al., 1984b; Sackett et al., 1985), were approximately 10-fold smaller than the critical concentrations of untreated tubulin. In the absence of subtilisin (Figure 1) or in the presence of PMSF-inactivated subtilisin, the fish tubulin failed to polymerize.[3] Thus, proteolysis by subtilisin stimulates the assembly of the piscine and mammalian tubulins to comparable extents.

In a second experiment, both polymerization and subunit cleavage were examined during incubation of *N. coriiceps* tubulin with subtilisin at 10 °C. Formation of stage I polymer (0−20 min in this experiment) corresponded

[3] The undigested brain tubulin control did not assemble at 5 °C because its critical concentration in MME + 1 mM GTP is substantially larger (unpublished observations) than that pertaining to PME + 1 mM GTP (Detrich et al., 1989, 1992).

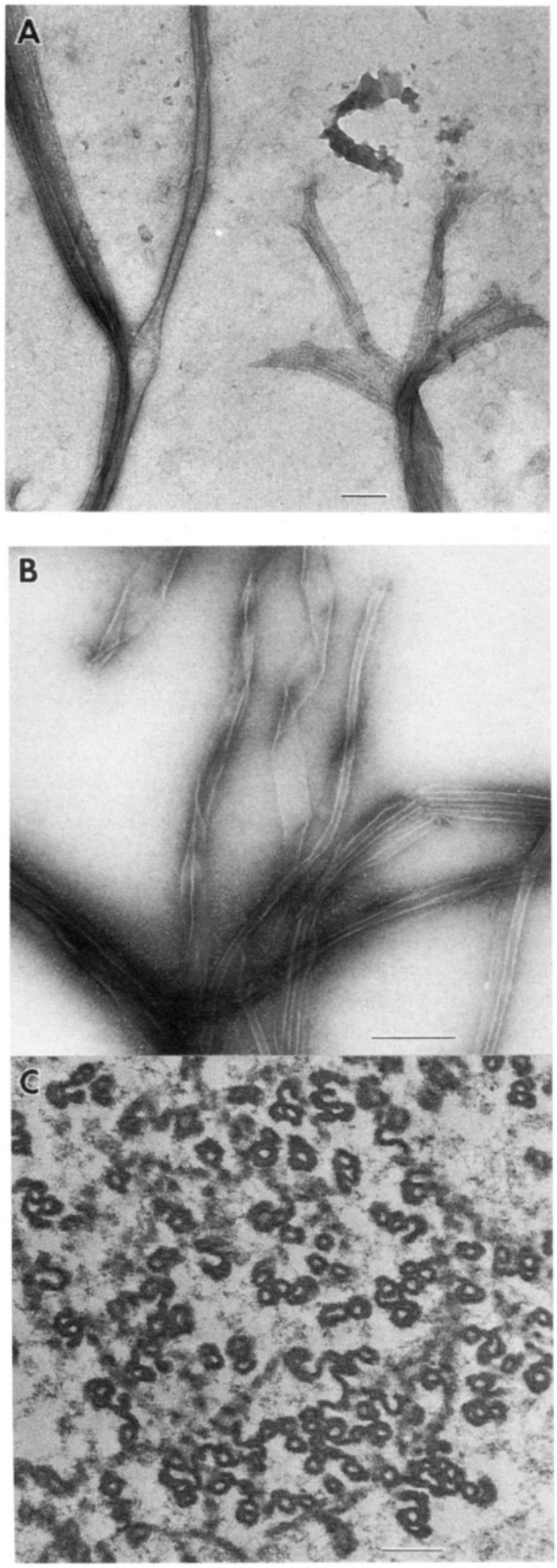

FIGURE 2: Morphology of subtilisin-induced polymers. (A) Negative-stain electron micrograph of stage I polymer formed at 5 °C. The grid was prepared 100 min after the addition of subtilisin (1% w/w) to tubulin (0.7 mg/mL). The bar represents 200 nm. (B) Negative-stain electron micrograph of stage II polymer (10 °C). The grid was prepared 200 min after addition of 1% (w/w) subtilisin to tubulin (0.8 mg/mL). The bar indicates 200 nm. (C) Thin-section electron micrograph of stage II polymer from the experiment shown in Figure 1 (5 °C). After assembly for 180 min, polymer was collected by sedimentation and prepared for thin-section microscopy. The bar represents 100 nm.

Table 1: Distribution of [^{14}C]GEE Adducts in Subunits and Proteolytic Fragments of *G. gibberifrons* Brain Tubulin

protease	gel band	fraction of label[a]
none	$\alpha + \beta$ (uncleaved)	1.0
	intact α	0.46
	intact β	0.54
subtilisin	48 kDa (from $\alpha + \beta$)	0.33[b]

[a] Tubulin contained 8−10 GEE-modified residues. The maximal uncertainty in these results is estimated to be ±5%. [b] $n = 3$.

temporally to cleavage of β subunits (Figure 3) to produce $\alpha\beta_s$ dimers [i.e., dimers with β chains shortened at their C termini by ~4 kDa; nomenclature is that of Bhattacharyya et al. (1985)]. Stage II assembly (>20 min) coincided with the slower, C-terminal cleavage of the α-tubulin monomers to α_s [α chains shortened at their C termini by ~4 kDa (Figure 3)], leading to a terminal digest containing a mixed population of $\alpha\beta_s$ and $\alpha_s\beta_s$ dimers (~50% each).

Two conclusions emerge from these studies: (1) the C termini of brain tubulins from Antarctic fishes and from mammals hinder polymerization at their respective physiological temperatures; and (2) removal of these acidic regions enhances the formation of polymers related structurally to microtubules. These observations suggest, but do not prove, that the C termini of the α and β tubulins of Antarctic fishes are not involved in cold adaptation of microtubule assembly.

Modification of Carboxyl Groups in Antarctic Fish Brain Tubulin. To investigate the role of carboxyl groups in the polymerization of the Antarctic fish tubulins, we employed a carbodiimide-catalyzed amidation reaction (Mejillano & Himes, 1991) to neutralize the side chains of glutamyl and aspartyl residues. Figure 4 shows the kinetics of the incorporation of [^{14}C]GEE groups into *G. gibberifrons* microtubules at 20 °C. (*Microtubules* were reacted with GEE in the presence of the carbodiimide to prevent modification of groups located at dimer−dimer contact regions.) The maximum stoichiometry of incorporation was 7 mol/mol of tubulin at a concentration of 0.1 M GEE and 12 at 0.3 M GEE. Using limited-proteolytic methods, we found that the GEE label was equally partitioned between the α and β subunits and that most (67%) of the modification occurred in the carboxy-terminal tails (Table 1). For comparison, we produced modified bovine brain tubulin containing ~12 GEE/dimer.

Figure 5 shows the effect of GEE modification on the polymerization of *G. gibberifrons* tubulin at two near-physiological temperatures. At 5 °C (Figure 5A) or 10 °C (Figure 5B), modified tubulin containing 12 GEE/dimer assembled more rapidly and at lower concentrations than did the control, unmodified protein. Similar results were obtained with modified and unmodified bovine tubulins at 37 °C (data not shown). By quantitative sedimentation (Figure 6, Table 2), we found that critical concentrations for assembly of GEE-modified fish tubulin at 5−10 °C were 2−3-fold lower than those of the control tubulin. Comparably modified bovine tubulin, with ~80% of the GEE adducts located in C-terminal peptides, assembled at 37 °C with a critical concentration that was 2.6-fold smaller than that for the control (Table 2). Energetic analysis showed that the change in the standard free energy of polymerization, $\delta\Delta G°$, upon GEE modification ranged from −0.4 to −0.6 kcal/mol for both piscine and bovine tubulins (Table 2). In contrast

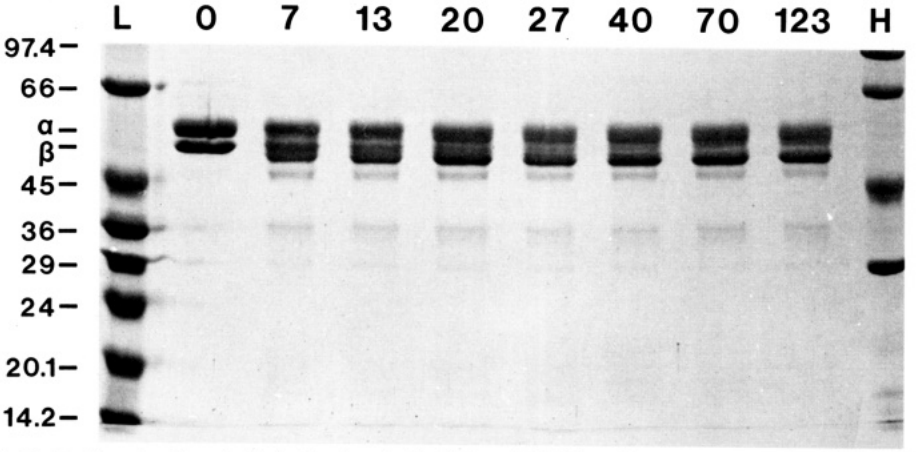

FIGURE 3: Kinetics of tubulin digestion by subtilisin. Brain tubulin (1.2 mg/mL) from *N. coriiceps* was incubated with 2% (w/w) subtilisin at 10 °C, and polymerization was monitored by turbidimetry. At intervals, aliquots were withdrawn, and proteolysis was terminated by the addition of PMSF. Intact (t = 0 min) and subtilisin-cleaved tubulins were examined by SDS−polyacrylamide gel electrophoresis on 9% slab gels (pH 9.2). Digestion intervals (see lane labels) were 7, 13, 20, 27, 40, 70, and 123 min. Lanes L and H contain low- and high-molecular-weight standards, respectively. The molecular weights (in thousands) of the standards and the positions of intact α- and β-tubulin chains are given on the vertical axis. Stage I polymerization (0−20 min in this experiment) corresponded to C-terminal cleavage of β chains to produce $\alpha\beta_s$ dimers, and stage II (>20 min) assembly coincided with the slower, C-terminal cleavage of α chains to yield $\alpha_s\beta_s$.

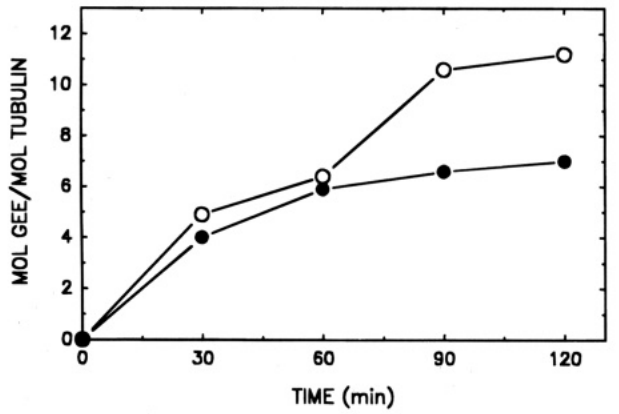

FIGURE 4: Kinetics of carboxyl group modification of Antarctic fish tubulin. Modification of the carboxyl groups of *G. gibberifrons* microtubules by carbodiimide-catalyzed amidation with glycine ethyl ester (GEE) was performed as described under Experimental Procedures. Two concentrations of GEE (●, 0.1 M; ○, 0.3 M) were employed. At intervals, aliquots of microtubules were withdrawn for analysis of GEE incorporation.

Table 2: Critical Concentrations and Energetics for Polymerization of GEE-Modified Brain Tubulins[a]

tubulin	treatment	T (°C)	C_c (mg/mL)	$\delta\Delta G°$ (kcal/mol)
G. gibberifrons	none	5	0.56	−0.6
G. gibberifrons	GEE	5	0.18	
G. gibberifrons	none	10	0.10	−0.4
G. gibberifrons	GEE	10	0.045	
Bos taurus (cow)	none	37	1.15	−0.6
B. taurus	GEE	37	0.44	

[a] Critical concentrations were measured by quantitative sedimentation as described under Experimental Procedures. Standard free energy changes for polymerization were calculated as $\Delta G° = -RT \ln K_A$, where R is the universal gas constant [1.987 cal/(mol·K)], T is the absolute temperature, and K_A (the association equilibrium constant for microtubule elongation) equals the reciprocal of C_c (C_c expressed in molarity) (Lee & Timasheff, 1977). At each temperature, $\delta\Delta G°$ was calculated as $\Delta G°_{GEE} - \Delta G°_{unmod}$, where $\Delta G°_{GEE}$ and $\Delta G°_{unmod}$ are the standard free energy changes for polymerization of GEE-treated and unmodified tubulins, respectively. GEE-modified tubulins contained 12 adducts/dimer.

to the polymorphs induced by subtilisin proteolysis (Figure 2), the polymers generated by the GEE-modified *G. gibberifrons* tubulin were largely microtubules indistinguishable from those produced by control tubulins (Figure 7). Thus, at temperatures near their physiological values, brain tubulins from an Antarctic fish and a mammal respond identically to carboxyl-group neutralization largely confined to their C termini. We conclude that the C termini of the Antarctic fish tubulins do not contain major molecular adaptations (e.g., reductions in content of acidic residues) that contribute to cold adaptation of microtubule assembly.

DISCUSSION

Charge Reduction and Cold Adaptation of Microtubule Assembly. The work presented here suggests strongly that the carboxyl termini of the α and β tubulins of Antarctic fishes are not sites of evolutionary adaptation of microtubule assembly to cold temperatures. Proteolytic removal of the C-terminal regions or neutralization of carboxyl groups, most of which are located in the C termini, enhance polymer formation by piscine or mammalian tubulins to comparable extents, within experimental error, at their respective physiological temperatures. Particularly striking is the equivalence of the changes in free energy of microtubule elongation, $\delta\Delta G°$, produced by modification of similar numbers of carboxyl groups in the two tubulins (Table 2). Thus, the C termini of Antarctic fish tubulins, like those of mammalian tubulins, probably inhibit microtubule assembly by generating charge−charge repulsion between neighboring subunits within the polymer. We cannot, however, exclude the possibility that C-terminal charge reduction serves as a mechanism of microtubule cold adaptation in other organisms.

One interpretative caveat concerns the partitioning of GEE moieties between the C-terminal tails and the 48-kDa fragments of the fish and bovine tubulins. For both tubulins, label was equally distributed between the intact α and β subunits (Table 1; Mejillano & Himes, 1991). However, *G. gibberifrons* tubulins modified at 8−10 carboxyl groups (20 °C) contained 67% of the adducts in the 4-kDa C-terminal subtilitic fragments (Table 1), whereas approximately 80% of the label in bovine tubulin (10 or 23 GEE/dimer, 37 °C)

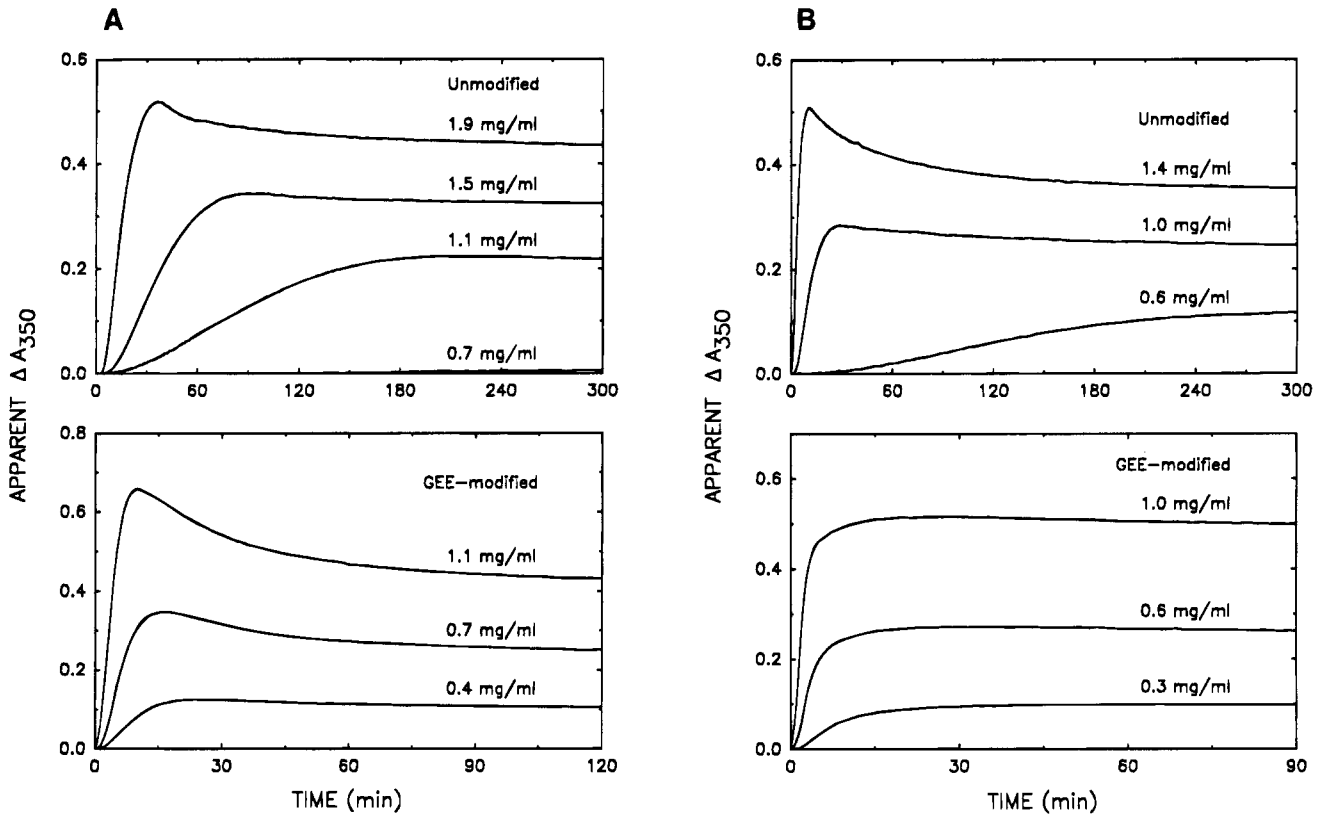

FIGURE 5: Polymerization of GEE-modified and unmodified brain tubulins from an Antarctic fish. Samples of GEE-modified (12 adducts/dimer; bottom panels of A and B) and control (top panels) tubulins from *G. gibberifrons* at the concentrations indicated were prepared at 0 °C in nucleotide-free PME buffer. GTP was added to each sample to a final concentration of 1 mM at zero time, and assembly was initiated by warming the samples from 0 to 5 °C (A) or 10 °C (B). Polymerization was monitored by the change in apparent absorbance at 350 nm (apparent ΔA_{350}).

was present in the C-terminal peptides (Mejillano & Himes, 1991). The C-terminal acidic residue content of one major Antarctic fish tubulin isotype, the neural β_{II} chain of the confamilial rockcod *N. coriiceps*, is equivalent to that of other vertebrate β chain isotypes (Detrich & Parker, 1993). Thus, the differential modification of the two tubulins might suggest that, under the modification conditions employed here, the C-terminal Glu and Asp residues of the Antarctic fish tubulin are less reactive toward the GEE nucleophile than are the corresponding bovine residues. Perhaps conformational and/or sequence constraints limit the reactivity of the C termini of Antarctic fish tubulins. Nevertheless, carboxyl-group neutralization, largely restricted to the C termini of piscine and bovine tubulins, produces similar energetic changes in microtubule assembly.

Although our data apparently rule out C-terminal charge reduction as an adaptive mechanism, we cannot exclude the possibility that charge reduction elsewhere within the dimer contributes importantly to the cold-adapted phenotype of the Antarctic fish tubulins. Both the α and β tubulins from brain tissues of Antarctic fishes are more basic than are tubulins from a temperate fish and a mammal, and native tubulin dimers from several Antarctic fishes express 1−2 fewer net negative charges than do the reference tubulins (Detrich & Overton, 1986, 1988). Amino acid analysis suggests that the increased basicity of the Antarctic fish tubulins is associated with a reduced content of acidic residues (Detrich & Overton, 1986, 1988), but substitution of basic residues for neutral and/or acidic residues in the primary sequence might also be involved. Furthermore, reduced posttranslational acetylation of α-tubulin could contribute to charge reduction, but preliminary observations suggest that Antarctic fish and bovine tubulins contain comparable amounts of acetylated α chains (Skoufias et al., 1988; unpublished results).

Polymer Formation by Modified Fish Tubulin. The polymers generated by proteolytically cleaved or GEE-modified Antarctic fish tubulins were generally similar to those observed with mammalian tubulins: *N. coriiceps* $\alpha_s\beta_s$ produced multiply hooked microtubules [cf. Serrano et al. (1984b, 1988)], and GEE-modified *G. gibberifrons* tubulin formed microtubules indistinguishable from unmodified tubulin [cf. Mejillano and Himes (1991)]. However, singly cleaved $\alpha\beta_s$ from *N. coriiceps* yielded mostly protofilament sheets rather than the hooked microtubules or rings described for mammalian $\alpha\beta_s$ tubulin (Bhattacharyya et al., 1985; Serrano et al., 1988). These differences may arise from factors intrinsic to the tubulin chains (e.g., distinct primary or tertiary structures of the β-chain C termini, leading to differential cleavage by subtilisin) or from external solution conditions [e.g., buffer composition, ionic strength, pH, nucleotide composition and magnesium concentration [cf. Peyrot et al. (1990)], and/or polymerization temperature].

Carboxy-Terminal Charge Status and Functional Specificity of Tubulins. Although probably not involved in cold adaptation of neural tubulin polymerization, C-terminal charge reduction, via differential posttranslational polyglutamylation, is likely to contribute to the functional differentiation of tissue-specific tubulins in vertebrates. For example, egg tubulin from *N. coriiceps*, which is composed of a small number of relatively basic isoforms, polymerizes more efficiently in vitro (i.e., with a 4−10-fold lower critical

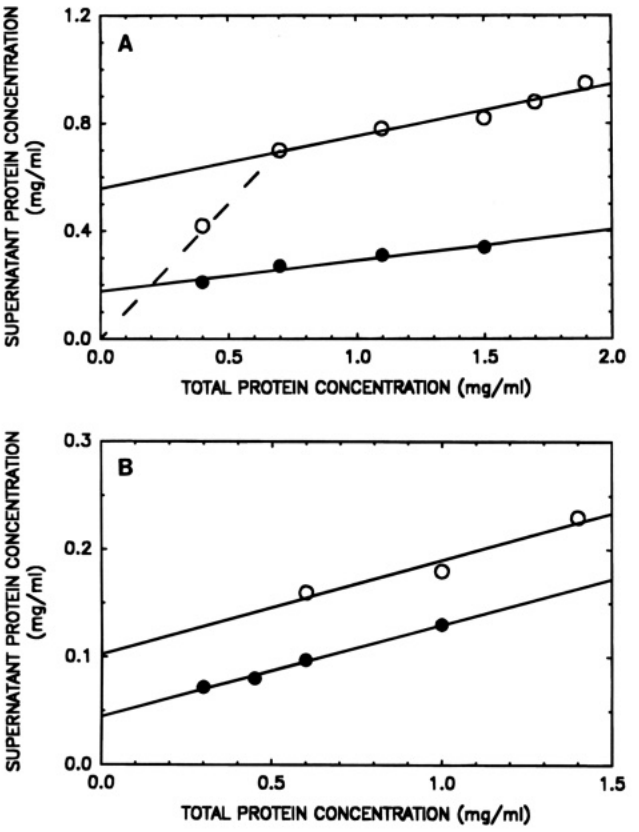

FIGURE 6: Critical concentrations for GEE-modified and unmodified brain tubulins from *G. gibberifrons*. Critical concentrations for polymerization of modified (●) and control (○) tubulins at 5 °C (A) and 10 °C (B) were measured by quantitative sedimentation. Samples of each tubulin at several concentrations were polymerized to steady state, and the polymer and monomer fractions were separated by centrifugation. Concentrations of the supernatant (monomer) fractions are plotted as a function of total tubulin concentration. For each tubulin, 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).

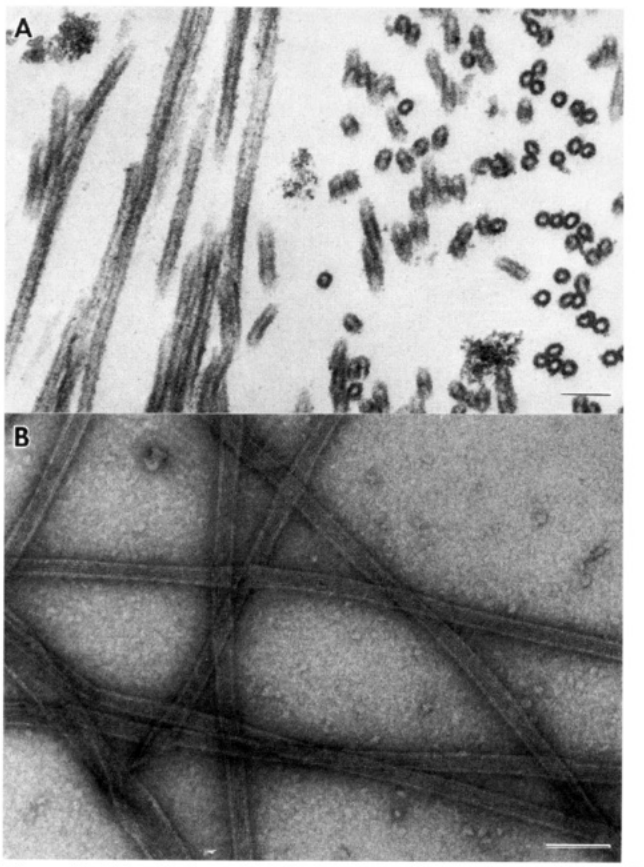

FIGURE 7: Morphology of polymers formed by GEE-modified and control tubulins from *G. gibberifrons*. (A) Thin-section electron micrograph of polymer formed by unmodified tubulin (1.1 mg/mL). After assembly for 300 min at 5 °C, polymer was collected by sedimentation and prepared for thin-section microscopy as described under Experimental Procedures. (B) Negative-stain electron micrograph of polymers formed by GEE-modified tubulin (12 residues modified). The grid was prepared 30 min after the start of assembly at 5 °C. The bar in each panel represents 100 nm.

concentration in the range 0−18 °C) than does the neural tubulin of this fish, probably due to reduced charge−charge repulsion conferred by decreased (or absent) C-terminal polyglutamylation (Detrich et al., 1992). Similarly, avian erythrocyte tubulins possess lower critical concentrations for polymerization than brain tubulins, due to the greater basicity and hydrophobicity of their single β_{VI} isotypes (Murphy et al., 1987; Murphy, 1991; Trinczek et al., 1993), to the absence of polyglutamylation of either chain type (Rüdiger & Weber, 1993), or both. We suggest that modification of neural, egg, and erythrocyte tubulins, using the enzymatic and chemical strategies employed here, followed by quantitative assessment of their polymerization energetics and/or dynamics should reveal the contribution of C-terminal charge to functional specialization of microtubule assembly.

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