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Selection of a Nucleation-Promoting Element following Chemical Modification of Tubulin†

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ABSTRACT: Following a 16-h incubation with a large excess of 2,5-hexanedione (2,5-HD) while in the assembled state, bovine brain tubulin contained a powerful nucleating component, the presence of which lowered the dissociation rate from 83 s^{-1} for untreated tubulin to 13 s^{-1} for 2,5-HD-treated tubulin. This nucleating component could be selectively concentrated by sequential stringent (conditions of low temperature and low tubulin concentration) cycles of assembly and disassembly. In 2-(*N*-morpholino)ethanesulfonic acid buffer without glycerol, the critical concentration of assembly of untreated tubulin (2.4 mg/mL) was 19 times higher than that of 2,5-HD-treated tubulin subjected to three sequential stringent cycles of assembly and disassembly (0.13 mg/mL). This highly nucleating 2,5-HD-treated tubulin preparation could both copolymerize with untreated tubulin and seed subcritical concentration assembly of untreated tubulin. Experiments to define the assembly-altering component have identified structural alterations to the α-tubulin monomer. While the α-tubulin subunit of native untreated tubulin dimer contained no chymotryptic cleavage sites, the native 2,5-HD-treated α-tubulin subunit was cleaved by chymotrypsin to yield a 37-kDa C-terminal fragment.

Cells are dependent on their cytoskeletons for a variety of their functions. Assembly and disassembly of microtubules into their component tubulin subunits are a necessity for many of these functions. Microtubule assembly and disassembly are required for the development of cell polarity in mouse blastomeres because the microtubules redistribute from the basal to the apical part of the cell (Houliston et al., 1988). During mitosis, the microtubules disassemble from their interphase configurations to assemble into the spindle (Vandre et al., 1984). Chromosome movement toward the spindle poles in metaphase depends upon disassembly of microtubules at their

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kinetochore ends (Gorbsky et al., 1987, 1988).

Microtubule assembly is a complex biochemical and thermodynamic process dependent on temperature, protein concentration, and nucleotide cofactors [for a review, see Dustin (1984)]. Tubulin subunits in microtubules have multiple interactions, both between and along protofilaments, adding to the complexity of microtubule assembly. Microtubule assembly in vitro is cooperative and displays saturation kinetics. Assembly begins with a *lag* or *nucleation phase*, indicative of the formation of stable microtubule nucleating seeds. Nucleation is followed by an *elongation phase*, representing addition of tubulin onto the protofilament ends, and then a *steady-state* plateau, when the net addition of tubulin onto growing microtubules equals the net loss of tubulin from shrinking microtubules.

Nucleation is a critical stage in microtubule assembly because (1) stable nuclei are needed for elongation and (2) the number of nuclei determines the overall elongation rate (Johnson & Borisy, 1977). Buffer components which impart structure to water (i.e., high concentrations of sodium glutamate or glycerol) also hydrate proteins, stabilize nuclei, and support spontaneous assembly of low concentrations of pure tubulin (Gekko & Timasheff, 1981; Arakawa & Timasheff, 1984). Without water-structuring buffer components, the assembly of low concentrations of tubulin (<1 mg/mL) has been achieved only with the support of microtubule-associated proteins or taxol (Dustin, 1984) or by the addition of nucleating elements such as centrosomes (Mitchison & Kirschner, 1984a).

Even though the first complete amino acid sequence of tubulin was published several years ago (Ponstingl et al., 1981; Krauhs et al., 1981), the three-dimensional structure of tubulin is largely unknown. Tubulin has yet to be crystallized, and supersecondary structural predictions largely arise by analogies to functional domains which tubulin shares with other proteins [for example, the guanosine 5′-triphosphate (GTP)[1] binding site (Sternlicht et al., 1987)]. Much of what is known about the way in which tubulin assembles into microtubules has been learned through chemical and proteolytic manipulation of tubulin. Lysine and arginine residues are postulated to play key roles in tubulin assembly since covalent modification of these residues inhibits assembly (Maccioni et al., 1981; Mellado et al., 1982; Szasz et al., 1982). Modification of lysine-394 of α-tubulin, which is readily methylated, reduces assembly competence by 50% (Szasz et al., 1986). Pure, monomeric α-tubulin is cleaved into major products of 40 and 18 kDa and minor products of 48, 44, 21, and 17 kDa by chymotrypsin (Yaffe et al., 1988). Three structural domains of each tubulin monomer have been identified by limited proteolysis (Serrano et al., 1984; Mandelkow et al., 1985; Sackett & Wolff, 1986). α-Tubulin has a 38- and a 17-kDa domain, with a trypsin cleavage site between the domains at arginine-339. Another domain of α-tubulin has been identified by proteolysis and antibody analysis (de la Vina et al., 1988). This extra domain is in the first third of the monomer. β-Tubulin has a 34- and a 21-kDa domain, with a chymotrypsin cleavage site between the domains at tyrosine-281. A 4-kDa domain on the C-terminus of each monomer is a likely regulatory site where microtubule-associated proteins bind to enhance assembly (Serrano et al., 1984). Chemical cross-linking studies of tubulin have identified some nearest-neighbor domain contacts in the tubulin heterodimer (Kirchner & Mandelkow, 1985; Serrano & Avila, 1985).

2,5-Hexanedione (2,5-HD) is the toxic metabolic product of *n*-hexane responsible for producing neuropathy and testicular atrophy in exposed animals (Krasavage et al., 1980). 2,5-HD binds to primary amines of proteins and cyclizes to form pyrroles (Katritsky et al., 1986; DeCaprio, 1987; Genter et al., 1987). Subsequent pyrrole autoxidation results in protein cross-linking (Graham et al., 1982; Szakal-Quin et al., 1986; DeCaprio, 1986). 2,5-HD-induced neurofilament cross-linking may explain the observed neurotoxicity (Graham et al., 1982; Garden et al., 1986) while tubulin cross-linking is proposed as the cause of the testicular atrophy (Boekelheide, 1987a).

Tubulin modification by reaction with 2,5-HD results in a functional alteration in microtubule assembly (Boekelheide, 1987b). Tubulin isolated from the brains and testes of rats intoxicated with 2,5-HD displays this assembly alteration (Boekelheide, 1987a). Tubulin treated in vitro with 2,5-HD assembles earlier and at a faster rate than untreated tubulin. Shorter but morphologically normal microtubules are produced. 2,5-HD treatment enables tubulin to polymerize under conditions of low temperature or low GTP concentration and causes the resulting microtubules to be resistant to depolymerization by cold.

The studies presented here further characterize tubulin treated with 2,5-HD and demonstrate that 2,5-HD modifies tubulin to produce a slowly dissociating tubulin population. This population nucleates microtuble assembly at low tubulin concentrations and low temperatures. The promotion of assembly is attributed to a modified monomeric form of tubulin. A prominent structural feature of this altered population is the modification of α-tubulin to expose extra proteolytic sites.

Materials and Methods

General. Protein concentrations were determined by a dye binding assay using the Bradford reagent (Bio-Rad, Rockville Centre, NY) with bovine serum albumin as the standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970) with 6 M urea in the sample buffer. Gels were stained by the silver technique (Merril et al., 1981) and scanned at 573 nm on a Gilford Response UV/Vis scanning spectrophotometer to estimate the protein content of bands. Molecular weight markers included bovine and egg albumin (66K and 45K), glyceraldehyde-3-phosphate dehydrogenase subunit (36K), carbonic anhydrase (29K), trypsinogen (24K), trypsin inhibitor (20.1K), and α-lactalbumin (14.2K).

A "cycle" of tubulin assembly and disassembly is defined by the following sequence: (1) warm temperature assembly, in the presence of 1 mM GTP, of tubulin (for purification, the assembly was at 37 °C; for experimentation with 2,5-HD-treated tubulin, the temperature varied); (2) centrifugation at that temperature at 100000*g* for 30 min; (3) resuspension of the microtubule-containing pellet in ice-cold buffer; (4) Teflon–glass homogenization by hand 3 times and incubation on ice for 30 min to disassemble the microtubules; (5) centrifugation at 4 °C at 100000*g* for 30 min to yield soluble, active tubulin in the supernatant. Tubulin resulting from step 5 is called C_nS (cycle 1, 2, 3, ..., supernatant); after 2,5-HD treatment, the tubulin is C_nSH.

[1] Abbreviations: 2,5-HD, 2,5-hexanedione; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GTP, guanosine 5′-triphosphate; C_nS and C_nSH, tubulin in 4 °C supernatant from *n* cycles of assembly/disassembly of untreated or 2,5-HD-treated tubulin, respectively; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DEAE, diethylaminoethyl.

Tubulin was purified according to Hamel and Lin (1981), with several modifications. Soluble proteins were isolated from bovine brain homogenates [100 g of brain to 75 mL of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.7, 1 mM EGTA, 0.5 mM $MgCl_2$, and 4 M glycerol] by centrifugation at 100000*g*. Tubulin was purified by binding to DEAE-Sephacel (equilibrated with 1 M sodium glutamate) in the presence of 0.1 mM GTP. After three washing steps with 1 M sodium glutamate containing 0.1 mM GTP, batch elution of tubulin from the Sephacel was achieved with 0.8 M NaCl in 1 M sodium glutamate with 0.1 mM GTP. One cycle of assembly/disassembly of this solution achieved C_1S, which was used for further purification and 2,5-HD treatment.

2,5-HD Incubation. Chemical modification was performed by using purified tubulin (C_1S) at 2 mg/mL in 1 M sodium glutamate, pH 6.6, with 1 mM GTP, incubated with 100 mM 2,5-HD (Eastman Kodak, Rochester, NY) at 37 °C for 16 h (Boekelheide, 1987b). The modified tubulin was then cycled to remove 2,5-HD and denatured tubulin. During this cycle, the warm centrifugation pellets were resuspended (step 3 above) in Mes buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.7, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM GTP], homogenized, and centrifuged. The resulting tubulin was named C_2SH and contained 1.5 2,5-dimethylpyrrole equiv per tubulin dimer (Boekelheide, 1987b). Untreated tubulin (C_2S) used as a control was also taken through a second purification cycle into Mes buffer. C_2SH was a mixture of at least five species of tubulin, including covalently cross-linked dimers and higher molecular weight multimers.

Assembly Analysis. Assembly was induced by placing ice-cold tubulin solutions of 0.5 mL into a prewarmed cuvette in a temperature-controlled water-jacketed cuvette holder of a Gilford 252 spectrophotometer set at 350 nm. Disassembly was induced by placing warm cuvettes with their assembled solutions into a precooled water-jacketed cuvette holder. Assemblies were analyzed for the following: V_{max}, the maximal velocity of the change in optical density per minute; tV_{max}, the time, in minutes, required for the assembly to reach V_{max}; and the maximal optical density after 30 min of assembly. The percent yield of tubulin in each assembly was defined as the ratio of the amount of tublin recovered from a cycle (step 5 above) to the total amount of tubulin in the original assembly mixture (step 1 above).

Electron Microscopy. In all instances, microtubule assembly was verified by placing a drop of the assembly mixture on a Formvar- and carbon-coated grid for 15–30 s. The grids were stained by dipping 3 times into 1% uranyl acetate and blotting dry. The samples were viewed in a Philips 410 transmission electron microscope. Microtubule samples were processed for quantitative electron microscopy according to Mitchison and Kirschner (1984b), except an equal volume of 1% glutaraldehyde in Mes buffer without GTP was added to the sample for 3 min and then 16 times the volume of 4 °C Mes buffer without GTP was added. Microtubules in 50 μL of each mixture were centrifuged onto Formvar-, carbon-, and polylysine-coated grids in a Beckman Airfuge for 5 min. Microtubule lengths were determined directly from electron microscopic negatives at 1677×. Magnifications were calibrated to a 1200 lines/mm diffraction line grating replica.

Critical Concentration. Measurement of the critical concentration of assembly in Mes buffer of C_2S and C_2SH was performed in triplicate while that of C_5SH was performed once. Various concentrations of C_2S, C_2SH, and C_5SH were assembled at 37 °C for 1 h and centrifuged at 100000*g* in a Beckman Airfuge for 3 min. The protein concentrations of the incubated solutions and supernatants were measured to determine the critical concentration (Gaskin et al., 1974).

Morphological Critical Concentration. The critical concentrations of C_2SH and C_5SH also were assayed morphologically. Various dilutions of C_2SH (range 0.9–0.14 mg/mL) and of C_5SH (range 0.75–0.0005 mg/mL) were incubated at 37 °C in covered tubes for 50 min. Aliquots (10 μL) were transferred for 10 min to Formvar- and carbon-coated grids prewarmed to 37 °C. The lowest protein concentration at which microtubules were observed by electron microscopy was taken as an estimate of the morphological critical concentration. C_2S was also analyzed by this method as a control.

Tubulin Assembly Rate Constants. The association (k_{on}) and dissociation (k_{off}) rate constants of C_2S and C_2SH in Mes buffer were determined by measuring the initial rates of polymerization or depolymerization, respectively, from sheared microtubule seeds as described by Johnson and Borisy (1977) with the theoretical modifications introduced by Carlier et al. (1984) and Mitchison and Kirschner (1984b). Microtubule seeds were prepared by shearing aliquots of 14 mg/mL solutions of assembled tubulin 3 times through a 21-gauge needle. Seed lengths were measured ultrastructurally and averaged 3.6 μm (n = 144) for C_2S and 1.3 μm (n = 142) for C_2SH. The mass concentration of the C_2S seeds was 1.6 mg/mL and of the C_2SH seeds was 1.4 mg/mL. C_2S seeds were added to C_2S, and C_2SH seeds were added to C_2SH prewarmed for 15 s at 37 °C. With a slight mixing delay (3–5 s), the assemblies were monitored spectrophotometrically. Linear regression analysis was used to produce the best-fit lines to the assembly and disassembly data.

Gel Filtration. C_3SH (9 mg in 1 mL of Mes buffer with 0.05% β-mercaptoethanol) was loaded onto a preequilibrated 0.9 × 50 cm Ultrogel AcA44 (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) column and eluted at 8 mL/h for collection into 0.8-mL fractions.

Aliquots containing 50 μg of protein from every other fraction were added to a solution of C_2S. The mixtures were brought to 0.5 mL with Mes buffer to make a final protein concentration of 1.85 mg/mL and assembled for 30 min at 37 °C. The fraction (number 13) which eluted before any detectable protein was used as a control.

Microtubules were processed for electron microscopy as described above except the glutaraldehyde and sample mixtures were diluted 13 times by 4 °C Mes buffer and the mixtures were centrifuged in the Airfuge for 10 min. The lengths of an average of 194 microtubules per fraction (range 72–312) were measured. The microtubule mass per assay was determined by adding the lengths of all the measured microtubules and correcting for background assembly, the actual area of the grid, the number of tubulin dimers per micrometer of microtubule [1625, per Amos and Klug (1974)], and the mass per tubulin dimer (100 kDa).

Limited Proteolysis and Immunoblotting. Limited proteolysis (Sackett & Wolff, 1986) of 0.8 mg/mL C_2S and C_4SH was performed at 8 and 10 °C. The protease concentrations were 5 μg/mL for typsin, chymotrypsin, and *Staphylococcus aureus* V8 protease and 10 μg/mL for subtilisin. At various times, samples were withdrawn, treated with protease inhibitors, and frozen in liquid nitrogen.

For immunoblotting, proteins were transferred from gels to nitrocellulose according to Gibson (1981), except 50 μg/mL chymotrypsin was used instead of Pronase. Primary antibodies were monoclonal anti-α- and anti-β-tubulins (Amersham Corp., Arlington Heights, IL) detected with affinity-purified biotinylated anti-mouse IgG (H + L) (Vector Laboratories,

Table I: Assembly Properties of 2,5-Hexanedione-Treated Tubulin during Multiple Cycles[a]

C_nSH assembled	protein (mg/mL)	temp (°C)	V_{max} (ΔOD_{350}/min)	tV_{max} (min)	ΔOD_{350} (at 30 min)	% yield (as $C_{n+1}SH$)
C_2SH	1.0	30	0.00040	16	0.010	2
C_3SH[b]	0.94	22.5	0.011	8.7	0.056	15
C_4SH	0.88	15	0.012	6.4	0.10	27

[a] All assemblies were performed in Mes assembly buffer with 1 mM GTP. Refer to Materials and Methods for explanations of column headings. [b] Often, C_3SH was obtained in too low a concentration to continue the cycles at 1 mg/mL. When this occurred, the C_3SH was frozen and used to resuspend the pellet of the C_2SH assembly the next time the procedure was performed. This manipulation allowed the 22.5 °C assembly to be performed at 1 mg/mL.

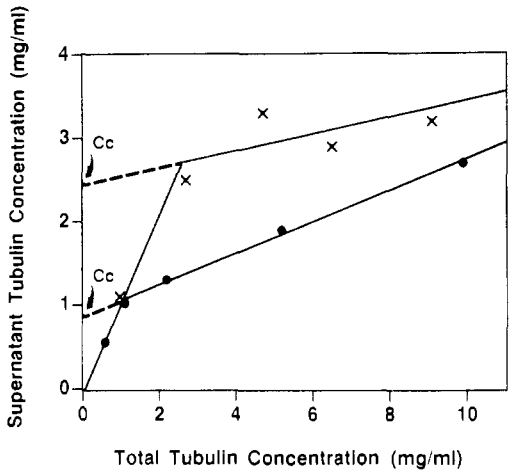

FIGURE 1: Critical concentration (Cc) measurement of C_2S (×) and C_2SH (●). Tubulin was assembled at various concentrations for 1 h and centrifuged at 100000*g*, and the protein concentrations of the supernatants were measured.

Burlingame, CA) visualized with the Vectastain ABC kit (Vector Laboratories) and 3,3′-diaminobenzidine (Towbin et al., 1979; Glass et al., 1981).

RESULTS

Critical Concentration of Assembly, Bulk Measurement. The critical concentrations for assembly of C_2S and C_2SH were measured in Mes buffer (Figure 1). C_2S had a critical concentration of 2.4 mg/mL, while C_2SH had a critical concentration of 0.86 mg/mL. Approximately twice as much assembly-incompetent C_2SH as C_2S was present (19% versus 9.3%), as indicated by the slopes of the graph. The critical concentration of C_2SH was confirmed as 0.87 mg/mL by an alternate method of plotting maximal optical density versus protein concentration (Gaskin et al., 1974).

Rate Constants of Assembly. The bulk association and dissociation rate constants (k_{on} and k_{off}) for assembly of C_2SH in Mes buffer were compared with those of C_2S. The rate constants were derived from the initial rates of polymerization or depolymerization from a known number of seeds of sheared microtubules (Figure 2). The k_{on} was determined from the dependence of the initial rate of polymerization upon protein concentration while the k_{off} was determined under depolymerization conditions from the initial rate of maximal depolymerization. C_2S behaved as expected (Carlier et al., 1984) with a rate of dissociation under depolymerization conditions faster than under polymerization conditions. However, C_2SH manifested a similar rate of dissociation under both polymerization and depolymerization conditions. The second-order rate constants, k_{on}, of the two preparations were very similar, 9.6×10^5 M^{-1} s^{-1} and 9.9×10^5 M^{-1} s^{-1} for C_2S and C_2SH, respectively. Consistent with the stability against cold disassembly displayed by 2,5-HD-treated tubulin (Boekelheide, 1987b), the first-order rate constants, k_{off}, demonstrated slowed disassembly of C_2SH relative to C_2S (13 s^{-1} versus 83 s^{-1}).

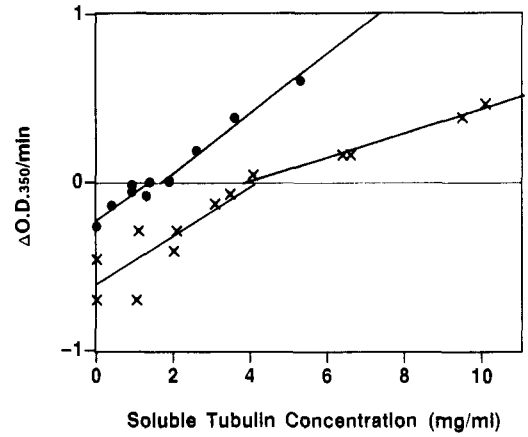

FIGURE 2: Plot of initial rates of (de)polymerization off sheared microtubule seeds versus added free tubulin concentration. The microtubule seeds of C_2S (×) and C_2SH (●) were present at 1.6 and 1.4 mg/mL, respectively, and had an average length of 3.6 and 1.3 μm, respectively. The slopes of the polymerization portion of the plot and the intercepts at the vertical axis were used to calculate the rate constants (for C_2S, 0.069 OD_{350} mL min^{-1} mg^{-1} and −0.62 OD_{350}/min; for C_2SH, 0.17 OD_{350} mL min^{-1} mg^{-1} and −0.23 OD_{350}/min).

Selection of the Nucleation-Promoting Component. Previous experiments identified a marked alteration in the assembly behavior of 2,5-HD-treated tubulin. When examined in the highly pro-assembly buffer 1 M sodium glutamate, 2,5-HD-modified tubulin assembled earlier and more rapidly than untreated tubulin and assembled at low temperatures (Boekelheide, 1987b).

An assembly condition which is stringent enough to allow only a small percentage of tubulin to polymerize could select for an exclusively assembling modified component of 2,5-HD-treated tubulin. Making the condition more stringent for each successive cycle could further concentrate the component. The most stringent assembly series of 2,5-HD-treated tubulin was performed at 1 mg/mL in Mes buffer, with the first assembly cycle with C_2SH at 30 °C, the second with C_3SH at 22.5 °C, and the third with C_4SH at 15 °C (Table I).

This stringent cycling resulted in C_5SH, highly pro-assembly tubulin capable of polymerization at 15 °C. C_5SH represented 0.03% of the original C_2SH and produced morphologically normal microtubules (Figure 3). The critical concentration of the stringently cycled C_5SH was determined to be 0.13 mg/mL with 13% assembly-incompetent tubulin.

Morphological Critical Concentration. Electron microscopy was used to test for the presence of a subpopulation of tubulin which assembled at concentrations below the bulk critical concentration of assembly. C_2S had no observable microtubules present below its bulk critical concentration, while C_2SH demonstrated rare, short, morphologically normal microtubules at concentrations as low as 0.14 mg/mL. The morphological critical concentration of C_5SH was more in agreement with the bulk critical concentration. The morphological critical concentration, estimated as the lowest concentration which

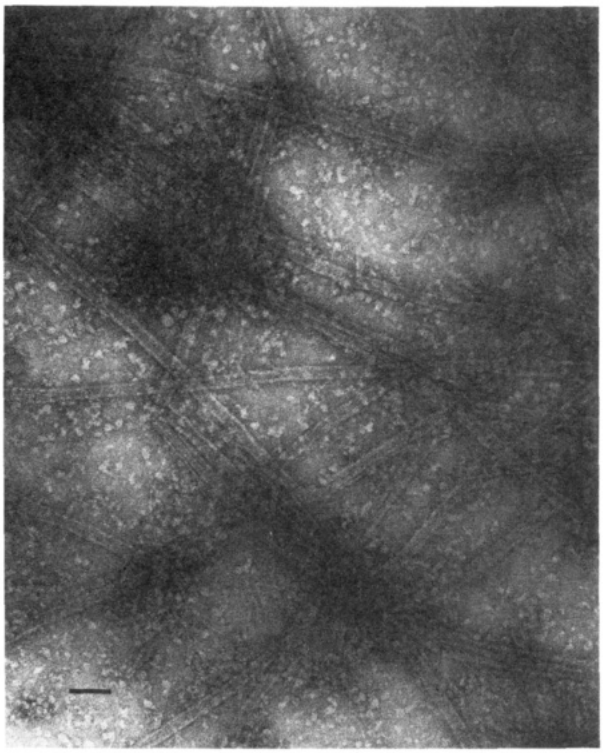

FIGURE 3: Electron micrograph of uranyl acetate stained microtubules derived from C_5SH assembled at 0.25 mg/mL at 37 °C. Bar = 1 μm.

would assemble into morphologically detectable microtubules at 37 °C, was 0.10 mg/mL (range 0.088–0.13 mg/mL, n = 3).

Nucleation and Copolymerization. Having determined that a pro-assembly component of 2,5-HD-treated tubulin could be progressively concentrated during multiple sequential cycles of stringent assembly, the nucleation and copolymerization capability of this material was investigated. In a seeding experiment, C_5SH was tested for its ability to nucleate assembly of C_2S below its critical concentration. A small amount of C_5SH (14.7 μL of a 2.3 mg/mL solution; 5% of the final tubulin content) was allowed to polymerize for 3 s on the edge of a 37 °C prewarmed cuvette prior to mixing with cold C_2S, giving a final tubulin concentration of 1.8 mg/mL (well below the 2.4 mg/mL critical concentration for assembly of C_2S). Under these conditions, C_5SH effectively seeded assembly of C_2S (Figure 4, curve a). C_2SH did not seed the assembly of this concentration of C_2S.

In a copolymerization experiment, C_5SH (final concentration 0.066 mg/mL, well below its critical concentration) was premixed on ice with C_2S (final concentration 1.8 mg/mL, well below its critical concentration) prior to assembly at 37 °C for 30 min. Little increase in optical density at 350 nm was observed with this assembly mixture; however, the rare C_5SH nucleated some assembly of C_2S, and morphologically normal microtubules were detectable by electron microscopy. In a second copolymerization experiment, C_5SH (5% of the final tubulin content) was premixed on ice with C_2S prior to assembly at 37 °C (final tubulin concentration, 3.7 mg/mL). This mixture was capable of rapid and extensive assembly (Figure 4, curve b). These nucleation and copolymerization assemblies can be compared with the slower polymerization exhibited by C_2S at a final tubulin concentration of 4.5 mg/mL (Figure 4, curve c).

Cold-induced disassembly of the seeded microtubules was used to investigate the potential redistribution of C_5SH in these

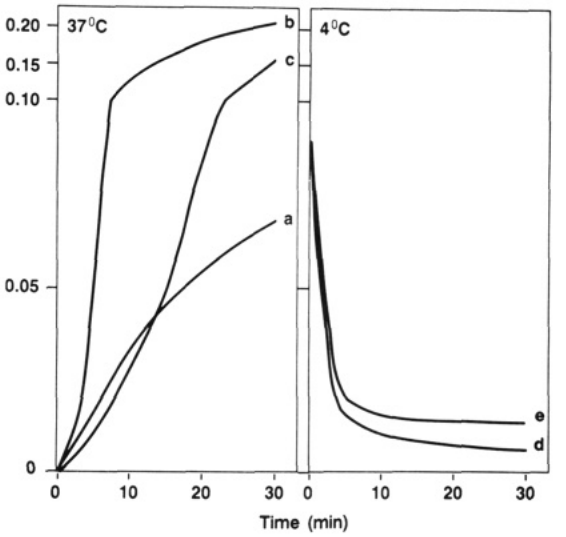

FIGURE 4: Assembly and disassembly behavior of C_2S following seeding or copolymerization by C_5SH. (Left) Assembly at 37 °C; (a) C_2S seeded with 5% C_5SH, final concentration 1.85 mg/mL; (b) C_2S mixed with 5% C_5SH, final concentration 3.7 mg/mL; (c) C_2S alone, 4.5 mg/mL. (Right) Disassembly at 4 °C [(d) disassembly after 30 min of assembly; (e) disassembly after 2 h of assembly] of microtubules from mixtures of C_2S seeded with 5% C_5SH at a final concentration of 1.85 mg/mL (assembly a). Note the change in scale on the vertical axis.

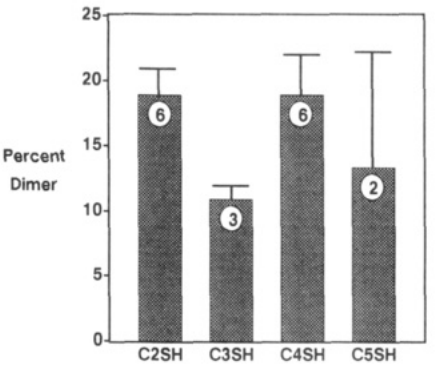

FIGURE 5: Compiled densitometry data (means and standard error) of the percentage of tubulin comprising the intermolecularly cross-linked dimer through cycles on silver-stained SDS–PAGE. Numbers in bars represent the number of experiments contributing to the data. Compiled scan data for other bands of 2,5-HD-treated tubulin showed a similar lack of variation.

microtubules at steady state. Disassemblies performed after the microtubules were assembled at 37 °C for 30 min and 2 h occurred at similar, rapid rates (Figure 4, curves d and e, respectively). Electron microscopy performed after 30 min of cold-induced disassembly revealed cold-stable microtubules in both samples. The lengths and the total mass of cold-stable microtubules per milliliter of the seeded mixtures were similar (5.1 μm and 0.51 ng for 30-min assembly versus 7.6 μm and 0.59 ng for 2-h assembly). No such cold-stable microtubules were detectable in the C_2S assembly.

Identification of the Assembly-Altering Component. The protein components present in each successive cycle of the stringent assembly series from C_2SH through C_5SH were analyzed by SDS–PAGE in an attempt to correlate the concentration of a given band (see Figure 7, lane 2) with the progressive increase in assembly ability. Densitometry with multivariate statistical analysis of silver-stained gels of the starting material and the warm supernatants from each assembly showed no alteration in band intensity with cycling (Figure 5).

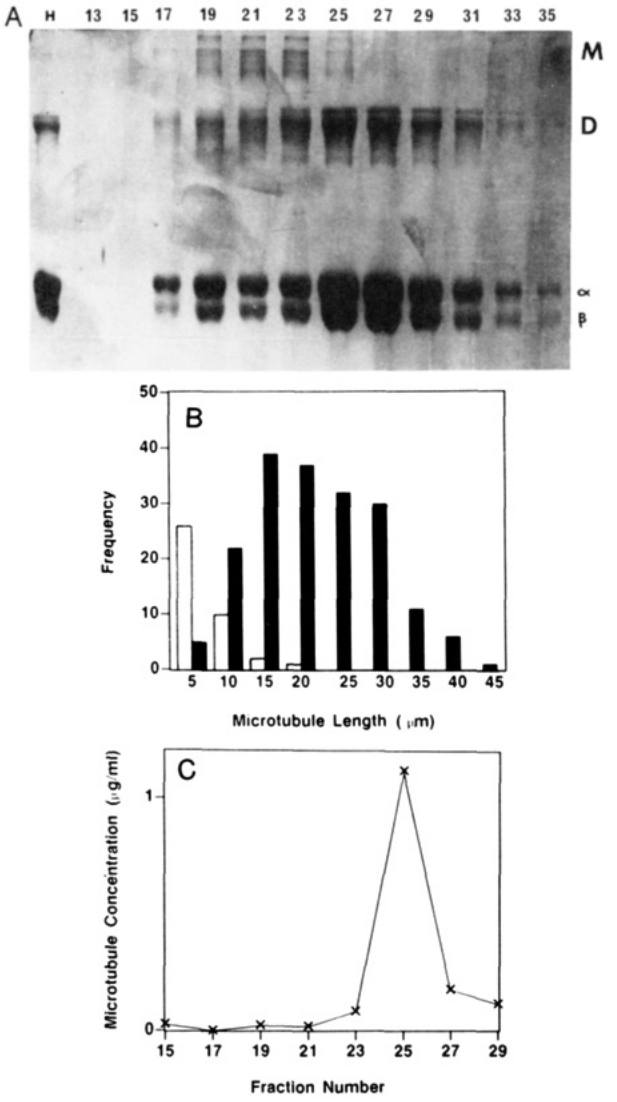

FIGURE 6: Gel filtration of C_3SH and copolymerization with C_2S. The 2,5-HD-treated tubulin loaded on the column (H) had undergone one assembly cycle at 2 mg/mL at 30 °C. (A) Odd-numbered fractions (13–35) were analyzed by SDS–PAGE using a silver-stained 7.5% polyacrylamide gel. M, high molecular weight covalently cross-linked multimers of tubulin; D, covalently cross-linked dimers of tubulin; α and β, α- and β-tubulin monomers. (B) Length distributions of microtubules assembled from mixtures of C_2S with 5% tubulin from fraction 21 (open bars) and fraction 25 (solid bars) at a final concentration of 1.85 mg/mL. (C) Total microtubule mass per milliliter of each mixture of C_2S with 5% tubulin from fractions at a final concentration of 1.85 mg/mL.

Gel filtration column chromatography was used to determine which component(s) of 2,5-HD-treated tubulin represented the assembly-altering element. Native C_3SH was eluted from the 0.9 × 50 cm Ultrogel AcA44 column with Mes buffer. The highest molecular weight component was effectively separated by this procedure (Figure 6A). A copolymerization assembly assay was employed to locate the assembly-altering component. A constant amount of protein from every other fraction (50 μg, 5% of the final tubulin content) was added to a constant amount of C_2S to reach a final protein concentration (1.85 mg/mL) below the critical concentration of C_2S. After incubation for 30 min at 37 °C, the lengths and total mass of microtubules induced to assemble by each fraction were determined by quantitative electron microscopy. The microtubule length distribution and mass data showed peak assembly and nucleating ability in fraction 25 (Figure 6B,C). Fraction 25 corresponded to the peak of monomeric-treated tubulin (present as a native 100-kDa dimer on the column) and two coeluting cross-linked dimers. The peak fractions of the highest molecular weight component (fractions 19–21) showed no nucleating activity.

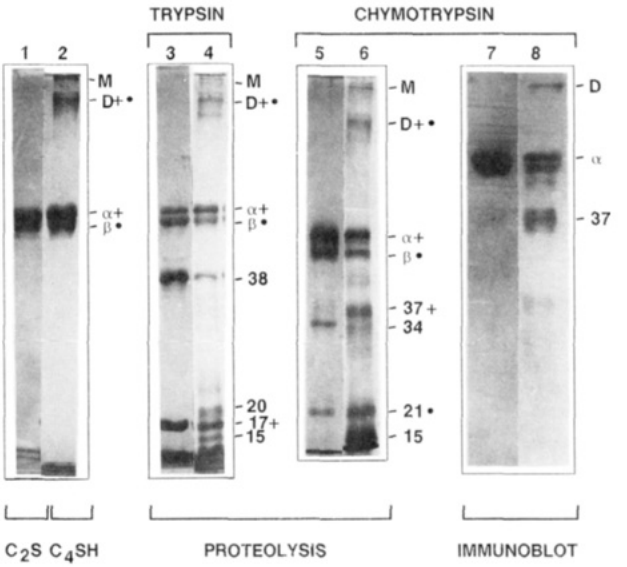

FIGURE 7: Limited proteolytic digestion of tubulin analyzed by SDS–PAGE using a silver-stained 10% polyacrylamide gel. Protein samples (0.8 mg/mL) were proteolyzed by 5 μg/mL chymotrypsin or trypsin at 10 °C. Lanes 1 and 2, C_2S, and C_4SH, respectively; indicated are bands M and D (described in legend to Figure 6A) and α- and β-tubulin; lanes 3 and 4, 30-min tryptic cleavage, major products (38, 17, 20, and 15 kDa); lanes 5 and 6, 60-min chymotryptic cleavage products (37, 34, 21, and 15 kDa). Indicated are bands which stained with anti-α-tubulin antibody (+) and anti-β-tubulin antibody (●). Lanes 7 and 8, anti-α-tubulin immunblot of 60-min chymotrypic fragments. C_2S (lane 7) and C_4SH (lane 8) were separated by SDS–PAGE on a 12% polyacrylamide gel prior to being transferred to nitrocellulose.

Table II: Untreated Tubulin (C_2S) and Multiply Cycled 2,5-Hexanedione-Treated Tubulin (C_4SH) Differ in Protease Sensitivity[a]

protease	major tubulin fragments (kDa)	
	C_2S	C_4SH
trypsin	38	38
		20 (α)
	17 (α)	17 (α)
		15
chymotrypsin		37 (α)
	34	34
	21 (β)	21 (β)
		15
Staph. aureus V8	36 (α)	36 (α)
	21 (α)	21 (α)
subtilisin	51	51
	47	47
	30	30

[a] Comparison of proteolysis results between untreated (C_2S) and 2,5-HD-treated tubulin whose nucleating component had been concentrated (C_4SH). Numbers represent estimated molecular masses in kilodaltons, while α or β in parentheses indicates staining with anti-α-tubulin or anti-β-tubulin antibody, respectively. Subtilisin cleavage was of C_5SH for up to 24 h at 8 °C. Cleavage of C_4SH α-tubulin by *S. aureus* V8 protease at 8 °C occurred at 3 times the rate as that of C_2S.

Limited Proteolysis and Immunoblot Analysis. Limited proteolysis was used to determine whether 2,5-HD treatment altered the native conformation of tubulin. C_2S and C_4SH were incubated at 10 °C at 0.8 mg/mL with 5 μg/mL trypsin or chymotrypsin. Electron microscopy confirmed that no microtubules were formed under these incubation conditions.

The results described below are pictured in Figure 7 and summarized in Table II.

As previously reported (Sackett & Wolff, 1986), trypsin selectively cleaved native untreated α-tubulin into two fragments of 38 and 17 kDa without proteolyzing β-tubulin as determined by densitometric analysis of silver-stained gels (Figure 7, lane 3) and immunoblot analysis (data not shown). Immunoblot analysis of the tryptic fragments of α-tubulin confirmed the report of Serrano et al. (1986) that the small, 17-kDa domain of α-tubulin was stained by anti-α-tubulin antibody. The 38-kDa cleavage band of C_4SH was never more than half as strong as in C_2S. This discrepancy in the intensity of the 38-kDa band may be explained by the presence of extra proteolytic bands (major, 20, 15 kDa; minor, 36, 34, 32, 30, 27, and 24 kDa) below 38 kDa in C_4SH (Figure 7, lane 4). By immunoblot analysis, no proteolytic β-tubulin bands were observed, and all the tryptic cleavage bands could be explained by α-tubulin cleavage, with the 36-, 24-, and 20-kDa bands, as well as the 17-kDa band, stained by anti-α-tubulin antibody. The 15-kDa band likely represents additional cleavage of the large domain of α-tubulin.

As previously reported (Sackett & Wolff, 1986), chymotrypsin selectively cleaved native untreated β-tubulin into two fragments of 34 and 21 kDa without proteolyzing α-tubulin as seen on silver-stained gels (Figure 7, lane 5) and immunblot analysis using monoclonal antibodies specific for α- and β-tubulin (Figure 7, lane 7). Immunblot analysis also confirmed the report of Kirchner and Mandelkow (1985) that the 21-kDa chymotryptic fragment of β-tubulin is recognized by anti-β-tubulin antibody (not shown). Chymotrypsin cleaved the β-subunit of C_4SH at a similar rate as C_2S as determined by densitometric analysis; however, extra proteolytic bands of 37 and 15 kDa were observed (Figure 7, lane 6). Densitometric analysis of the silver-strained gels demonstrated the expected decrease in β-tubulin and an unanticipated decrease of α-tubulin in the 2,5-HD-treated sample. Immunblot analysis identified the 37-kDa band as well as minor bands at 53 and 48 kDa as products of α-tubulin cleavage (Figure 7, lane 8).

The discovery that the small domain of α-tubulin was stained by the anti-α-tubulin antibody identifies the major immunostained chymotryptic fragment (37 kDa) of α-tubulin produced during 2,5-HD-treated tubulin proteolysis as a portion of the large domain attached to the small domain (Figure 7, lanes 6 and 8). Therefore, the remaining 15-kDa chymotryptic fragment of α-tubulin was a cleavage product of the large domain (Figure 7, lane 6).

S. aureus V8 protease cleaved α-tubulin of both C_2S and C_4SH in two places, as previously described (de la Vina et al., 1988). However, these α-tubulin cleavages occurred at a greater rate in the 2,5-HD-modified tubulin (Table II). By densitometric analysis, α-tubulin was cleaved 3 times as fast in C_4SH as compared to C_2S with a concomitant increase in the rate of appearance of the 36- and 21-kDa degradation products. Limited proteolysis with subtilisin, which cleaves a 4-kDa C-terminal fragment from both α- and β-tubulin (Serrano et al., 1984), was unaltered after concentration of the assembly-altering component (Table II).

Discussion

The generation of "2,5-HD-treated tubulin" required that tubulin be reacted with 2,5-HD while in the *microtubule* form. Attempts to modify unpolymerized tubulin in solution (by adding calcium to inhibit assembly or performing the reaction in Mes buffer at tubulin concentrations which did not support assembly) resulted in assembly-incompetent tubulin without production of an assembly-enhancing tubulin component. Given the requirements of the assembly enhancement for oxygen-dependent cross-linking (Boekelheide, 1987b) and for reaction of 2,5-HD with tubulin in the assembled state, the enhancement can be explained by *chemical fixation* of tubulin into a pro-assembly conformation. That both glutaraldehyde and dimethyl suberimidate can produce assembly enhancement (unreported observations) further supports an underlying requirement for tubulin lysyl ϵ-amine cross-linking as the primary 2,5-HD assembly-altering treatment effect.

One can draw analogies between the effect of 2,5-HD, subtilisin cleavage, and taxol on tubulin assembly. Subtilisin-cleaved tubulin assembles into polymers at a lower critical concentration than untreated tubulin (Bhattacharya et al., 1985; Serrano et al., 1988). However, subtilisin-cleaved tubulin assembles into morphologically abnormal forms, while 2,5-HD-treated tubulin forms the usual microtubule polymer. In addition, subtilisin cleavage of tubulin was not altered by 2,5-HD treatment. Taxol enables microtubules to polymerize under low temperature or low GTP conditions and causes them to be resistant to calcium-induced depolymerization (Manfredi & Horwitz, 1984). Taxol stabilizes associations between tubulin dimers, thus reducing the lag time of assembly with an increased number of stable nuclei. In addition, taxol may fix tubulin into the same conformation produced by the shift in temperature which initiates assembly (Hamel et al., 1981). A difference between taxol and 2,5-HD is that taxol binds tubulin noncovalently and 2,5-HD binds tubulin covalently. The covalent binding of 2,5-HD to tubulin lends the modified structure to more rigorous structural analysis than can be possible with taxol modification.

The elucidation of the kinetic properties underlying 2,5-HD-induced assembly enhancement was facilitated by the use of the Mes buffer system. The relatively low support of assembly provided by this buffer exaggerated the differences between 2,5-HD-treated tubulin and C_2S. In Mes buffer, the critical concentration for assembly of C_2S was 3 times that of C_2SH. 2,5-HD treatment decreased the dissociation rate of tubulin assembly 6-fold without altering the association rate. The lower critical concentration of assembly and decreased dissociation rate can be explained by the presence of 2,5-HD-modified tubulin subunits with "pro-assembly" character. The rate of assembly is enhanced by these modified subunits which slowly dissociate from, and thus stabilize, microtubule nuclei. The rate of disassembly in C_2SH is slowed by interpenetration of the slowly dissociating altered component throughout the microtubules and intermittent capping of the ends.

Stringent assembly conditions during multiple sequential cycles were used to concentrate the altered component. Under the most stringent conditions employed, a 2,5-HD-treated tubulin with remarkably enhanced nucleating capability was produced. C_5SH had a critical concentration for assembly at 37 °C in Mes buffer of 0.13 mg/mL, almost 7 times less than the critical concentration of C_2SH and 19 times less than the critical concentration of C_2S. With C_5SH, the selection procedure lowered the bulk critical concentration to the morphological critical concentration at which a subpopulation of the original 2,5-HD-treated tubulin assembled. No conclusion about the homogeneity of the resulting tubulin can be made, however, until the strength of the functional alteration is known.

C_5SH readily copolymerized with C_2S and nucleated C_2S assembly below its critical concentration. Cold-induced disassembly of untreated microtubules seeded by C_5SH demonstrated the presence of many short, cold-stable microtubule

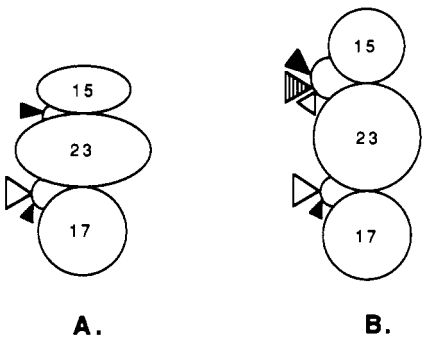

FIGURE 8: Model of 2,5-HD-modified α-tubulin. Proteolytic cleavage sites for trypsin (open arrowheads), chymotrypsin (hatched arrowhead) and *S. aureus* V8 protease (solid arrowheads) are indicated for C_2S (A) and 2,5-HD-treated tubulin with pro-assembly character (B). As indicated by an increased rate of *S. aureus* V8 protease cleavage and the apperance of new tryptic and chymotryptic cleavage sites, a more open three-domain structure is proposed for the assembly-enhancing 2,5-HD-modified tubulin. Numbers refer to molecular weights, $\times 10^{-3}$, of major fragments identified in proteolytic data.

cores. The presence of similarly sized and similar total masses of the cold-stable cores after 30 min and 2 h of assembly shows that a constant amount of C_5SH failed to redistribute at steady state, by depolymerization and repolymerization, throughout the microtubule. In contrast, studies with C_2S in a similar buffer system indicated a complete redistribution of core tubulin by 90 min at steady state (Kristofferson et al., 1986). Therefore, the C_5SH core microtubules described in the present study were composed of truly nondissociating tubulin which was not disassembling and participating in the dynamic assembly process.

The reaction between 2,5-HD and microtubules produced a diverse population of derivatized and cross-linked tubulin products, some of which could be visualized as α- and β-tubulin monomers and intermolecularly cross-linked tubulin dimers and multimers on denaturing SDS–PAGE. The ability to progressively concentrate the assembly-altering component was consistent with its being a unique product of 2,5-HD treatment and provided a technique to correlate assembly enhancement with a structural element. Densitometric analysis of polyacrylamide gels of the 2,5-HD-treated tubulins produced during sequential stringent cycling showed no component to be concentrated or lost during this selection procedure. Components may be maintained during cycling for one or both of the following reasons: (1) more than one component contained the specific assembly-enhancing modification, and each was carried through the cycles in constant proportions; (2) inactive components were carried through cycles through nonspecific association with microtubule walls (Yaffe et al., 1988). A third alternative, that 2,5-HD-treated tubulin monomers were continuously being cross-linked into native intermolecularly cross-linked tubulin multimers which thus appeared as a fairly constant proportion of the assembly mixtures, was eliminated experimentally (data not shown).

Some components can be disregarded as the assembly-enhancing element. The assembly behavior of the components after partial separation by gel filtration eliminated the possibility that the highest molecular weight multimer was the assembly-enhancing element. Nonetheless, this component was maintained as a constant proportion of the assembly mixture, possibly because of nonspecific associations with native tubulin and microtubules (reason 2 above). The gel filtration fractions with maximal nucleating activity contained the α- and β-tubulin monomers (eluting from the column as native tubulin dimer) and coeluting intermolecularly cross-linked dimers. Various chromatographic methods have failed to separate the native covalently intermolecularly cross-linked dimer from noncovalently associated tubulin dimer. If one of the intermolecularly cross-linked dimers represented the sole assembly-enhancing element, then sequential stringent cycling should have produced a visible enhancement of the relatively low protein content of the corresponding band in SDS–PAGE. Since no such band enrichment occurred, these data indicate a modified tubulin monomer as an assembly-enhancing element. Covalently intermolecularly cross-linked dimer may contain some specific modifications leading to the assembly alteration, as in reason 1 above, but the intermolecular cross-link itself does not cause the effect.

Evidence from the limited proteolysis and immunoblot experiments supports the conclusion that a modified α-tubulin subunit was progressively concentrated during sequential stringent cycling of 2,5-HD-treated tubulin. Specifically, the large domain of α-tubulin was modified by 2,5-HD treatment. Untreated α-tubulin was cleaved by trypsin at a single site between the large and small domains and was resistant to chymotrypsin. Coincident with concentration of the 2,5-HD-induced assembly-enhancing component during sequential stringent cycling, α-tubulin was modified such that the large domain became susceptible to selective trypsin and chymotrypsin proteolysis. The presence of these extra proteolytic sites indicated a more open structure of, or the formation of two domains from, the large domain of α-tubulin (Figure 8).

While most studies of unassembled tubulin show a two-domain structure (Mandelkow et al., 1985; Sackett & Wolff, 1986), a three-domain structure can be identified with some proteases (de la Vina et al., 1988). Three-dimensional X-ray fiber diffraction analysis of assembled microtubules indicates a three-domain repeat electron density structure along protofilaments (Beese et al., 1987a,b). If the tubulin-to-microtubule transition represents a conformational shift from a predominantly two-domain state to a three-domain state, then chemical fixation of the assembled-state structure by microtubule incubation with 2,5-HD would explain both the alteration in proteolytic digestion and the enhanced assembly of the modified tubulin.

Future determination of the locations of 2,5-HD adducts and further study of the conformational modifications induced by 2,5-HD treatment will help to elucidate the tertiary interactions required for the tubulin-to-microtubule transition.

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