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Expression of a Human α-Tubulin: Properties of the Isolated Subunit†

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ABSTRACT: We examined the in vitro expression and biochemical properties of the isolated α subunit of tubulin both in rabbit reticulocyte lysates and in *Escherichia coli* extracts. Both systems produce soluble, full-length human α-tubulin polypeptide. When α-tubulin mRNA is translated in rabbit reticulocyte lysates, the isolated α subunit is *fully* functional as assayed by coassembly with bovine brain tubulin using temperature-dependent or taxol/salt assembly procedures. The conformation of the isolated α subunit was probed by limited proteolytic digestion with chymotrypsin and by reductive methylation. Limited proteolysis studies indicated that the "monomeric" α subunit is highly susceptible to chymotrypsin digestion and becomes resistant to chymotrypsin cleavage following incorporation into the heterodimer. Reductive methylation indicated that the unassociated α subunit has a highly reactive lysyl residue essential for microtubule assembly similar to that observed in the heterodimer. In contrast, α-tubulin expressed in *E. coli* lysates was incapable of coassemblying with bovine brain tubulin. Differences in assembly competence of the two α-tubulin products appear to be related to formylation of the N-terminal methionine in the procaryotic synthesized subunit. These findings suggest that the amino-terminal methionine of α-tubulin plays an essential role in the isolated subunit and/or in the heterodimer, a hypothesis supported by chemical reactivity studies [Sherman, G., Rosenberry, T. L., & Sternlicht, H. (1983) *J. Biol. Chem. 258*, 2148–2156] which imply that this residue is in a salt-bridge interaction in the dimer.

Microtubules are filamentous polymer structures involved in a variety of diverse functions in eucaryotic cells. Mitosis, morphogenesis, and maintenance of cell shape are some of the processes dependent on the ordered assembly and disassembly of microtubules (Dustin, 1984). A molecular understanding of these functions will involve in part a detailed knowledge of the properties of the constituent heterodimer protein of the microtubule, tubulin, and its two homologous subunits, α- and β-tubulin. These two subunits, each ~450 amino acid residues in length (Luduena et al., 1977; Ponstingl et al., 1981; Krauhs et al., 1981), are held together by noncovalent interactions (Lee et al., 1973) with an apparent K_D of ~1 μM at 4 °C (Detrich & Williams, 1978). Despite this relatively high K_D, it has not yet been possible to isolate the native subunits from the heterodimer (Kirchner & Mandelkow, 1985; H. Sternlicht et al., unpublished observations), and, therefore, even such basic properties as stability and assembly competence of the isolated subunits are not known.

As an alternative approach to probe the biochemical properties of the individual subunits, we have used expression vectors to obtain native, isolated α- and β-tubulin. In previous work, we expressed a human α-tubulin cDNA (Cowen et al., 1983) in *Escherichia coli*, thereby producing significant amounts of α-tubulin (>1% of total protein) (Yaffe et al., 1986, 1987). However, the protein was insoluble and accumulated in "midbodies". [Similar findings were also obtained by Wu and Yarbrough (1986) in their investigation of *Trypanosoma rhodesiense* α- and β-tubulin expression in *E. coli*.] In this study, we describe the use of in vitro expression systems for the synthesis and characterization of the isolated α-tubulin subunit. In vitro systems typically generate soluble proteins (albeit at low level concentrations, ≤10 nM). This is an important consideration in the case of tubulin as no procedure currently exists to generate the native polypeptides from insoluble or denatured products. In this paper, we examine and evaluate two systems for the in vitro expression of human α-tubulin: a eucaryotic translation system using rabbit reticulocyte lysates and a procaryotic coupled transcription–translation system using *E. coli* lysates. Both systems produce full-length α-tubulin subunit. We demonstrate that the α subunit synthesized in reticulocyte lysates is stable and able to form assembly-competent heterodimers with bovine brain tubulin. In contrast, the α subunit synthesized in *E. coli* lysates is unable to coassemble into microtubule polymer, an effect which we attribute to *N*-formyl modification of the amino-terminal methionine residue in the *E. coli* lysates.

We have a long-standing interest in the C-terminal domain of α-tubulin, a region potentially involved in dimer–dimer contacts in the microtubule (Kirchner & Mandelkow, 1985). In the free heterodimer, this region contains a highly reactive lysyl residue, Lys-394, whose methylation renders tubulin assembly-incompetent (Sherman et al., 1983; Szasz et al., 1986). The enhanced reactivity of this residue as a nucleophile is attributed to its location in a cluster of basic residues (Blank

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et al., 1986; Szasz et al., 1986). In this study, we use reductive methylation and limited proteolysis to probe the conformation of the isolated α subunit. We show that the unassociated α subunit has a highly reactive lysyl residue essential for microtubule assembly similar to that observed in the heterodimer.

Materials and Methods

Materials. L-Methionyl-L-arginine (Met-Arg, U.S. Biochemicals) was N-formylated following Sheehan and Yang (1958) and confirmed by ^{1}H NMR. *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)[1]-treated trypsin (Sigma) was purified by reverse-phase HPLC according to Titani et al. (1982). Chymotrypsin was purchased from Worthington Biochemicals and assayed with insulin β-chain (Titani et al., 1982). Taxol, a gift from the National Cancer Institute and Flow Laboratories, Inc., was prepared as a 4 mM stock solution in DMSO, aliquoted, and frozen at −20 °C. [^{35}S]-Methionine (>1200 Ci/mmol) was purchased from Amersham. Autoradiography was performed with Kodak X-Omat R X-ray film.

Preparation of Microtubule Protein. Microtubule protein (MTP) was isolated by repetitive cycles of assembly–disassembly from bovine brains following a procedure (Sternlicht & Ringel, 1979) modified from Gaskin et al. (1974). Two-cycle preparations [~85% tubulin and ~15% microtubule-associated proteins (MAPs)] were stored at −20 °C in PB + 5 M glycerol (PB+5MG) buffer (Sternlicht & Ringel, 1979).

DNAs and Enzymes. Kα1, a recombinant derivative of pBR322 containing a cDNA with the entire coding region of an α-tubulin gene from a human keratinocyte cell line (Cowan et al., 1983), was a gift from Dr. Don Cleveland. pKK223-3, a vector for the regulated expression of foreign genes in *E. coli* (Brosius & Holy, 1984), as well as M13mp18 and M13mp19 was purchased from Pharmacia. Gemini riboprobe vector pGEM3 and T7 RNA polymerase were purchased from Promega Biotec. Restriction and DNA-modifying enzymes were purchased from Boehringer Mannheim Biochemicals, BRL, IBI, or New England Nuclear. Standard manipulations of DNA were performed as described in Maniatis et al. (1982) unless otherwise indicated.

Antibodies. The supernatant form of the rat monoclonal anti-α-tubulin antibody YL1/2 (Kilmartin et al., 1982) was purchased from Accurate Chemical Co. The mouse monoclonal anti-β-tubulin antibody DM1B (Blose et al., 1984) was purchased from Amersham.

SDS–PAGE. Proteins were electrophoresed on 9% polyacrylamide–SDS gels (Laemmli, 1970) unless indicated otherwise.

Plasmid Isolation and Purification. Plasmid DNAs were isolated from D1210 by alkaline lysis (Birnboim & Doly, 1979) followed by CsCl density gradient centrifugation. Ethidium bromide was removed by chromatography on Dowex AG-50Wx8 resin, and CsCl was removed by dialysis against TE buffer (Maniatis et al., 1982).

Construction of pKKHαT 11/2. Kα1 was cut in the 5′-noncoding region with *Bst*EII and digested with *Bal*31 (slow isoenzyme form, 1 unit/25 pmol of ends) to remove varying amounts of 5′-noncoding DNA upstream from the initiation codon. End repair was accomplished with T4 DNA polymerase and the DNA cleaved 3′ to the coding region with *Hin*dIII.

pKK223-3 was cut in the pUC-8 polylinker region with *Eco*RI, the 5′ overhang was converted to a blunt end with DNA polymerase I (Klenow), and the DNA was then cleaved with *Hin*dIII. Vector and insert sequences were ligated at 15 °C in 10-μL volumes containing 0.5 unit of T4 DNA ligase and the ligation products used to transform D1210, a lac I^q derivative of HB101 (Sadler et al., 1980), by the procedure of Hanahan (1983). Replica plated transformants were screened by colony hybridization (Grunstein & Hogness, 1975; Hanahan & Meselson, 1980) and/or immunoreactivity (Helfman et al., 1982) following induction with isopropyl β-D-thiogalactoside (IPTG). Further details of this construction and screening procedure will be described elsewhere.

Coupled Transcription–Translation Reactions. In vitro synthesis of α-tubulin from pKKHαT 11/2 was performed at 37 °C for 1 h in 30- or 60-μL coupled transcription–translation reactions (Amersham) according to Collins (1979) and Chen and Zubay (1983). Reaction mixtures contained (per 30 μL) 30 μCi of [^{35}S]methionine, 5 μg of supercoiled DNA, and 5 μL of S-30 supernatant and were supplemented with 0.6 mM IPTG to prevent transcriptional inhibition (Chen & Zubay, 1983).

Construction of pGEM3α10. Kα1 was partially digested with *Pst*I and cut to completion with *Hin*dIII (cf. Figure 1). The 1.5-kb α-tubulin cDNA insert was subcloned into the multiple cloning site of pGEM3 and transformed into D1210. In this orientation, T7 polymerase transcribes sense α-tubulin mRNA. Recombinant clones were selected by hybridization to a nick-translated probe (Nilson et al., 1983B) prepared from a 1.4-kb kα1 *Pst*I fragment. Plasmid DNA was purified as described above and sequenced (Sanger et al., 1977) using an oligonucleotide primer complementary to the T7 promoter.

Transcription Reactions. pGEM3α10 (5 μg) was digested with *Hin*dIII (Figure 1) and the linearized template used to direct transcription of α-tubulin mRNA in 100-μL reactions containing 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 0.5 mM each of ATP, GTP, CTP, and UTP, and 50 units of T7 RNA polymerase (Promega). Reactions were incubated at 37 °C for 60 or 120 min, and the RNA (15–45 μg) was recovered by ethanol precipitation.

In Vitro Translations. α-Tubulin mRNA (1–2 μg) was translated in 50-μL reactions containing 35 μL of micrococcal nuclease treated rabbit reticulocyte lysates (Promega), 20 μM unlabeled amino acids, and 75 μCi of [^{35}S]methionine for 1 h at 30 °C.

Immunoprecipitations. Immunoprecipitations were performed as modified from Cleveland and Kirschner (1981) using the rat anti-α-tubulin antibody YL1/2 (Kilmartin et al., 1982) or the mouse anti-β-tubulin antibody DM1B (Blose et al., 1984). The in vitro translations (5–7 μL) were diluted in PBS and incubated with various concentrations of antibody (45-μL final volume) for 90 min at room temperature. (In the case of YL1/2, a rat monoclonal with low affinity for protein A, this primary incubation was followed by an additional 90-min secondary incubation with rabbit anti-rat IgG.) Twenty microliters of a 10% *Staphylococcus aureus* suspension (Pansorbin, Calbiochem) was added, and the mixtures were

[1] Abbreviations: PB, a microtubule protein stabilizing buffer (pH 6.7) consisting of 0.1 M MES, 2 mM EGTA, 0.1 mM EDTA, 2 mM mercaptoethanol, and 0.5 mM $MgCl_2$; MTP, microtubule protein; MAPs, microtubule-associated proteins; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactoside; kb, kilobase(s); DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodalton(s); PPO, 2,5-diphenyloxazole.

incubated at 4 °C for 30 min. The precipitated proteins were washed 3 times with PBS containing 0.05% NP-40 and resuspended in 80 μL of 2× gel loading buffer (Laemmli, 1970), and 35-μL samples were analyzed by SDS–PAGE. Gels were processed for fluorography as described by Bonner and Laskey (1974).

Anion-Exchange HPLC Purification of α-Tubulin. In vitro synthesis reactions (procaryotic and eucaryotic) were diluted with 3 volumes of PB+4MG buffer, supplemented to 3 mg/mL BSA, 1 mM PMSF, 40 μg/mL leupeptin, and 10 μg/mL each of DNase I and RNase A, and incubated for an additional 15 min at their respective synthesis temperatures. Unincorporated radiolabel and low molecular weight contaminants were removed by Sephadex G-25 chromatography, and samples were loaded on a Mono-Q anion-exchange HPLC column (Pharmacia). Proteins were eluted with a 40-min linear gradient of 0–1 M NaCl in 20 mM sodium phosphate, pH 6.8, at a flow rate of 1 mL/min. Fractions were collected at 0.5-min intervals directly into 1 mL of PB+2.5 MG containing 0.2 mM GTP. Aliquots were removed for scintillation counting, and fractions containing α-tubulin were pooled and dialyzed against PB+2.5MG containing 0.1 mM GTP for 1 h at 4 °C.

Chymotrypsin Proteolysis. Limited chymotrypsin digestions were performed on HPLC-purified ("monomeric") α-tubulin synthesized in rabbit reticulocyte lysates. The monomeric α-subunit was supplemented with BSA (1–2 mg/mL) or mixed with MTP or phosphocellulose-purified tubulin and carried through one cycle of assembly/diassembly (1.2–3 mg/mL final concentration). The samples were then digested with chymotrypsin (1:20–1:250 w/w) for 15–30 min either at room temperature or at 37 °C. Digests were analyzed by electrophoresis through 15% SDS gels, stained with Coomassie blue, and autoradiographed.

Preparation of ^{3}H-Tyrosylated Tubulin. MTP was radiolabeled by addition of [^{3}H]tyrosine at the penultimate position of the α chain using tubulin tyrosine ligase. Crude ligase extracts were isolated from the first warm supernatant fractions of bovine brain tubulin preparations by DEAE-cellulose chromatography following Flavin et al. (1982). Clarified MTP in PB+2.5MG was supplemented to 25 mM Tris-HCl, pH 7.2, 100 mM KCl, 10 mM DTT, 2.5 mM ATP, and 12.5 mM $MgCl_2$ and incubated with 20% (v/v) crude tubulin tryosine ligase in the presence of 10 μCi of [^{3}H]tyrosine (7 μM final concentration) for 1 h at 37 °C. Unincorporated radiolabel was removed by dialysis against PB+2.5MG.

Preparation of Denatured Bovine Brain α-Tubulin. Denatured α-tubulin, used as a negative control in the copolymerization studies, was isolated from dual-isotope (^{3}H and ^{14}C) reductively methylated, carboxamidomethylated MTP (Szasz et al., 1986). The α chain was electroeluted from preparative SDS–PAGE gels, lyophilized, extracted in 8 M urea, and dialyzed extensively against 0.02 M sodium phosphate, pH 6.8.

Copolymerization by Temperature-Dependent Microtubule Assembly. In vitro synthesized α-tubulin (crude or HPLC purified) was mixed with carrier MTP (3.5–4 mg/mL final concentration) in PB+2.5MG, and microtubule assembly was initiated by the addition of 1 mM GTP. Samples were incubated at 37 °C for 40 min, and the microtubules were pelleted through beds containing 3 volumes of 50% sucrose in PB at 150000*g* for 60–90 min. Pellets were resuspended in half the starting volume of PB+2.5MG over 30 min at 4 °C, and denatured or undepolymerized protein was removed by centrifugation at 80000*g* for 20 min. This assembly/disassembly process was repeated for a total of three cycles.

Copolymerization by Taxol-Dependent Microtubule Assembly. HPLC-purified, in vitro synthesized α-tubulin was mixed with carrier MTP in PB+2.5MG and induced to assemble into microtubules as described above. After 30 min, microtubules were stabilized by the addition of 50 μM taxol. MAPs were displaced by bringing the samples to 0.5 M NaCl followed by incubation for an additional 20 min. Microtubules and MAP aggregates were pelleted through 50% sucrose cushions, resuspended at 4 °C in half the starting volume of PB+2.5MG, supplemented to 12 mM $CaCl_2$ (Dinsmore & Sloboda, 1987), and clarified as described above. Assembly was reinitiated by addition of 15 mM EGTA, 1 mM GTP, and 20 μM taxol, and additional cycles of assembly/disassembly were performed in an analogous manner.

Amino-Terminus Determination. Amino-terminus determinations were performed by using a procedure based on reductive methylation (Sherman et al., 1983; Haas & Rosenberry, 1985). ^{35}S-Labeled procaryotic (180 μL) and eucaryotic (150 μL) synthesis reaction products were mixed with 380 μg of MTP and purified by anion-exchange HPLC. Samples were denatured in 6 M guanidine hydrochloride, extensively methylated with 6 mM formaldehyde and 72 mM sodium cyanoborohydride for 30 min at 37 °C, and dialyzed exhaustively against 1 mM ammonium bicarbonate. Methylated protein samples were dried in vacuo and hydrolyzed in sealed tubes under N_2 in 6 N HCl containing 80 mM β-mercaptoethanol for 18 h at 125 °C. Hydrolysates were analyzed on a Beckman Model 119CL amino acid analyzer using a W-3H resin (0.6 × 20 cm) and a three-buffer system (Mays & Rosenberry, 1981). Fractions were collected at 0.46-min intervals for scintillation counting.

Tryptic Digestion of ^{35}S-α-Tubulin. In vitro synthesized procaryotic and eucaryotic α-tubulins (100–150-μL crude reaction volumes) were purified by anion-exchange HPLC and supplemented with MTP to a final specific activity of 34000 cpm/mg. Cysteines were reduced with 10 mM DTT and alkylated with 20 mM *N*-ethylmaleimide for 15 min at 37 °C. Protein samples were precipitated with 5 volumes of ice-cold ethanol, resuspended in 50 mM HEPES, pH 8.0, to a final concentration of 1 mg/mL, and digested with trypsin (75 μg/mg of protein) at 37 °C for 4 h. Digests were chromatographed on a Synchropak RP-P C18 column (0.46 × 25 cm, 300-Å pore size, Synchrom) using a 1-h linear gradient of 0–60% acetonitrile in water with 0.1% trifluoroacetic acid at a flow rate of 1 mL/min.

RESULTS

In Vitro Expression of Human α-Tubulin. In order to study the isolated α-tubulin subunit, we constructed the expression vectors pGEM3α10 and pKKHαT 11/2 shown in Figure 1. These vectors were derived from Kα1, a plasmid containing the complete coding sequence of a human keratinocyte α-tubulin cDNA (Cowan et al., 1983), and parent expression plasmids pGEM3 and pKK223-3 (Brosius & Holy, 1984), respectively. pGEM3α10 directs the transcription of α-tubulin mRNA by T7 RNA polymerase. pKKHαT 11/2 uses the tac promoter and was previously shown to generate insoluble α-tubulin during in vivo expression in *E. coli* (Yaffe et al., 1986; manuscript in preparation). In this study, we used both vectors to synthesize soluble radiolabeled α-tubulin in cell-free protein synthesis reactions. These expression systems were then compared for the efficiency of transcription/translation and the production of functional α-tubulin subunits.

pGEM3α10 is capable of the in vitro transcription of large amounts of α-tubulin mRNA using T7 RNA polymerase

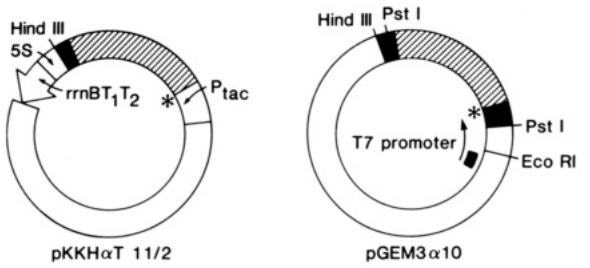

FIGURE 1: α-Tubulin expression vectors pGEM3α10 and pKKHαT 11/2. Hatched and black areas denote the α-tubulin coding sequence and 5′- and 3′-noncoding regions, respectively, from Kα1, the parent α-tubulin cDNA plasmid. Asterisks denote the initiating ATG codon. "P_{tac}" and "5S" in pKKHαT 11/2 denote, respectively, the tac promoter and a part of the 5S ribosomal RNA sequence which contains two strong RNA transcription terminators (rrnBT_1 and T_2). Further details are given under Materials and Methods.

(Materials and Methods). When the uncapped α-tubulin mRNAs were translated in nuclease-treated rabbit reticulocyte lysates supplemented with [^{35}S]methionine, ~0.5–1.0 nM radiolabeled 55-kDa polypeptide was produced per 1–2 μg of mRNA in a 50-μL reaction volume. This polypeptide was the major protein product constituting greater than 80% of total radiolabeled protein (Figure 2A, lane 2). When the in vitro synthesis products were purified by anion-exchange HPLC, the 55-kDa polypeptide eluted as a sharp radiolabeled peak at 0.6 M NaCl, at the identical elution position as dimeric tubulin standards from bovine brain (Figure 2B). We estimated from autoradiographic analysis (cf. Figure 6B) that ~40% of the total synthesized α-tubulin is recovered in the HPLC-purified fraction; similar recoveries were observed with native tubulin controls.

Grasso (1966) showed that rabbit reticulocytes contain essentially no microtubules, suggesting that the concentrations of tubulin in these cells are low. Consequently, we expected that α-tubulin synthesized in these cell lysates should exist as monomeric subunits rather than in dimeric association with residual β-tubulin. Indeed, incubation of the reticulocyte lysate reaction crudes with an anti-β-tubulin monoclonal antibody (Blose et al., 1984) failed to immunoprecipitate any radiolabeled α-tubulin (Figure 2, lane 4) although this antibody readily immunoprecipitated ^{3}H-tyrosylated α-tubulin in dimer controls (data not shown). If the synthesized α-tubulin were complexed with residual reticulocyte β-tubulin, it should have been coimmunoprecipitated by the anti-β-tubulin antibody. Furthermore, the in vitro synthesized α subunit gave a limited chymotrypsin digestion pattern different from α-tubulin in the heterodimer whereas after coassembly with MTP it gave an identical digestion pattern (see below, Figure 7).

pKKHαT 11/2 produced soluble α-tubulin in vitro using a coupled transcription/translation system prepared from *E. coli* lysates. When S-30 extracts from *E. coli* strain MRE 600 were supplemented with [^{35}S]methionine and programmed with 5 μg of supercoiled pKKHαT 11/2 plasmid DNA, ~20–100 pM 55-kDa radiolabeled polypeptide was produced per 30-μL reaction mixture. In addition, smaller amounts of lower molecular weight polypeptides as well as ~8–40 pM β-lactamase, the ampicillin-resistance gene product, were produced (Figure 3A). Under these conditions, greater than 90% of the synthesized α-tubulin was soluble as judged by ultracentrifugation. When the procaryotic in vitro synthesis products were purified by anion-exchange HPLC, the 55-kDa polypeptide eluted at 0.6 M NaCl (peak II, Figure 3B). Although a minor peak consisting of apparent breakdown products was detected upon purification of the reaction crude (peak I, Figure 3B), pulse–chase experiments indicated that the 55-kDa polypeptide was stable to degradation for at least 3 h in the reaction mixture (data not shown).

For equivalent amounts of plasmid DNA, pGEM3α10 produces >200-fold more α-tubulin than pKKHαT 11/2. Both systems produce 55-kDa polypeptides that correspond to full-length human α-tubulin as indicated by the following: (i) comigration of the 55-kDa products with α-tubulin from bovine brain on SDS–PAGE (Figures 2A and 3B); (ii) elution of the 55-kDa products during anion-exchange chromatography at a position (0.6 M NaCl) expected for polypeptides

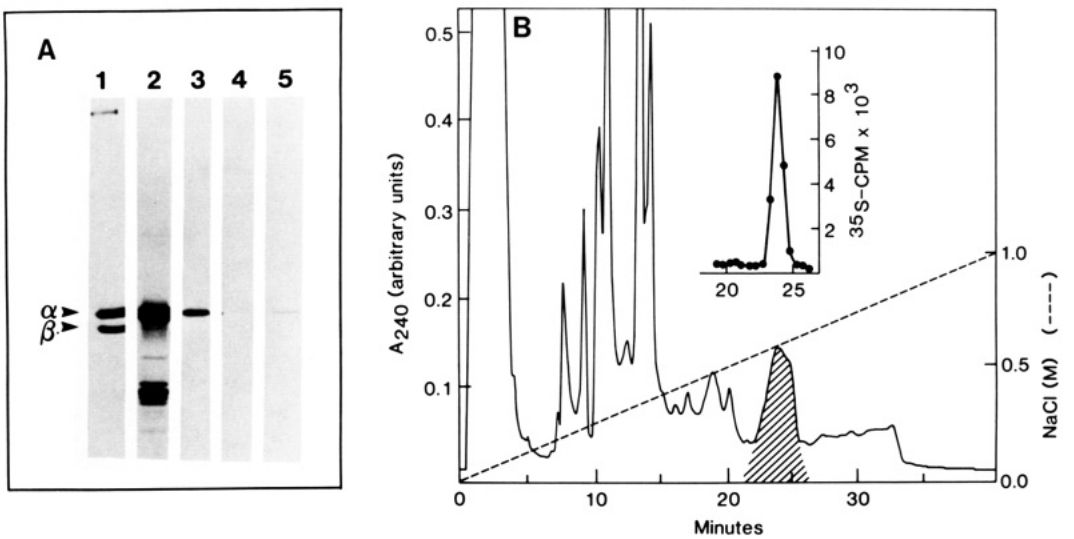

FIGURE 2: In vitro expression of α-tubulin using pGEM3α10. (A) α-Tubulin mRNA (2 μg) transcribed from pGEM3α10 using T7 RNA polymerase was translated in 50 μL of rabbit reticulocyte lysates containing [^{35}S]methionine (Materials and Methods). Following translation, aliquots were removed and analyzed by SDS–PAGE and autoradiography (lane 2), or immunoprecipitated with (lane 3) or without (lane 5) the anti-α-tubulin antibody YL1/2, a monoclonal antibody specific for the C-terminal residues (Wehland et al., 1984), or immunoprecipitated (lane 4) with the anti-β-tubulin antibody DM1B (Blose et al., 1984). Lane 1 contains radiolabeled MTP markers. (B) The translation product crude obtained as described in (A) was treated with DNaseI and RNaseA. Unincorporated radiolabel and low molecular weight contaminants were removed by chromatography on Sephadex G-25, and the sample was then supplemented with MTP carrier and immediately loaded on a Mono-Q anion-exchange HPLC column (B). Proteins were eluted with a linear gradient (---) of 0–1 M NaCl. α-Tubulin eluted as a single sharp peak (inset) with tubulin dimer (hatched region) at ~0.6 M NaCl. Identical elution positions were observed for α-tubulin in both the presence and absence of MTP carrier. An autoradiogram of the purified subunit is shown in lane 4 of Figure 6B.

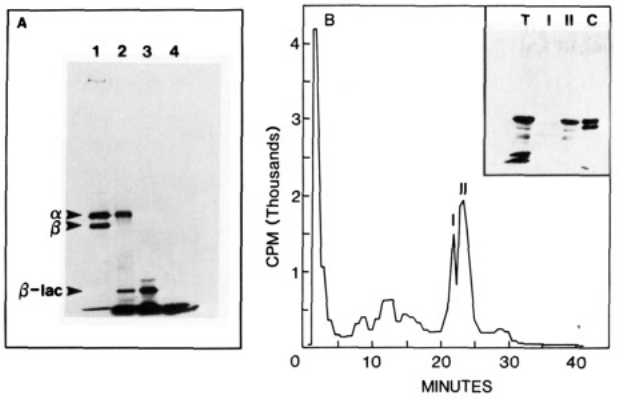

FIGURE 3: In vitro expression of α-tubulin using pKKHαT 11/2. 30-μL coupled transcription–translation reactions containing 5 μL of S-30 supernatant, 31 μCi of [^{35}S]methionine, and 5 μg of supercoiled plasmid DNA were incubated at 37 °C for 60 min. (A) 3-μL aliquots were analyzed by SDS–PAGE. Gels were dried and autoradiographed for 72 h at −70 °C. Lane 1, ^{14}C-methylated MTP markers; lanes 2, 3, and 4, translation products using pKKHαT 11/2, pAT153 (a pBR322 derivative), or no exogenous DNA template, respectively. (B) the ^{35}S-labeled crude reaction products (inset, lane T) were treated with DNase and RNase and purified by anion-exchange chromatography. In vitro synthesized α-tubulin eluted at ~0.6 M NaCl (peak II) as shown by autoradiography of SDS gels (inset, lane II). The peak II fractions were pooled, immediately dialyzed at 4 °C against PB+2.5MG buffer supplemented with 0.1 mM GTP, and used in coassembly studies (cf. Figure 5A). Lane C of the inset shows ^{14}C-methylated MTP markers. Note that unlike the eucaryotic isolation which gave an α-tubulin free of breakdown products (cf. Figure 2 inset; also lane 4 of Figure 6B), peak II, while predominantly the 55-kDa polypeptide, contained small amounts of breakdown products. These breakdown products appeared to be different than those in peak I (inset).

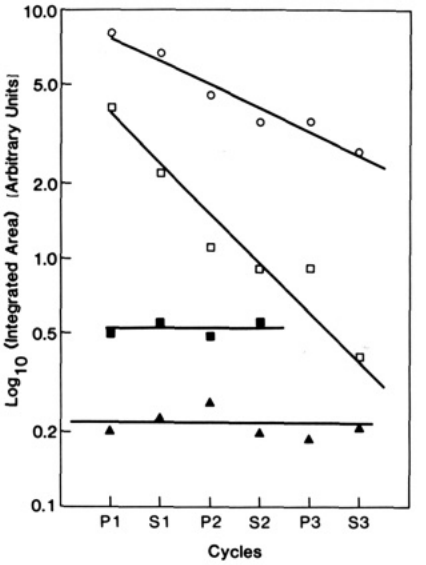

FIGURE 4: Coassembly studies using temperature-dependent assembly/diassembly. ^{35}S-labeled crude reaction products derived from pKKHαT 11/2 were mixed with MTP carrier and subjected to three cycles of temperature-dependent assembly. Aliquots containing equal amounts of total protein were removed at pellet (P) and supernatant (S) stages of each cycle and analyzed by SDS–PAGE and autoradiography. The intensity of the procaryotic α-tubulin (○) and β-lactamase bands (□) in each sample was quantitated by laser densitometry and compared with the corresponding specific activities of a ^{3}H-tyrosylated tubulin control (▲). In addition, we show the corresponding specific activities of the α-tubulin derived from pGEMα10 (■). To facilitate comparisons, the specific activities of the ^{3}H control sample (500 cpm/mg) and the eucaryotic-based α-tubulin (11 300 cpm/mg) at P1 were arbitrarily set to 0.2 and 0.5, respectively, and the specific activities of the remaining samples were normalized accordingly.

containing the highly charged C-terminal region of α-tubulin (Figures 2B and 3B); (iii) tryptic digestion patterns which are consistent with fully synthesized protein (Figure 10 below); (iv) ability to be immunoprecipitated by the monoclonal antibody YL1/2 specific for the last three C-terminal residues of α-tubulin (Figure 2A for pGEM3α10; data not shown for procaryotic study).

Assembly Studies. To determine if the synthesized α-tubulins were functional, we assayed their ability to coassemble into microtubules with bovine brain microtubule protein. These studies surprisingly established that α-tubulin produced in the procaryotic lysates was *nonfunctional*, in contrast with α-tubulin produced in the eucaryotic system which was *functional* in both temperature- and taxol-dependent coassembly assays.

Procaryotic reaction crudes from pKKHαT 11/2 were incubated for 90 min at 37 °C with phosphocellulose-purified tubulin, then supplemented with MTP, and carried through three cycles of temperature-dependent assembly/disassembly. The β-[^{35}S]lactamase cosynthesized with α-tubulin in these procaryotic lysates served as an internal negative control. [^{3}H]Tyr^{451}-tubulin, uniquely radiolabeled at position 451 in the α subunit with tubulin tyrosine ligase (Flavin et al., 1982), served as a positive control and was carried in parallel. The [^{3}H]tubulin controls assembled and disassembled at constant specific activity through the various cycles in contrast to both the procaryotic ^{35}S-α-tubulin and β-[^{35}S]lactamase samples which showed a progressive decrease in specific activity (Figure 4). At the end of the third cycle, the ^{35}S-α-tubulin specific activities in the supernatants, for example, were a factor of ~3 lower than their initial values in the first pellet. Although this decrease in specific activity was significantly less than that observed for β-lactamase (Figure 4), it was nevertheless a large decrease as judged by the behavior of the [^{3}H]tubulin control.

In order to determine whether this coassembly behavior might be due to the presence of contaminants in the *E. coli* lysates, we performed similar coassembly studies with the purified α-tubulin fraction isolated by anion-exchange chromatography (peak II, Figure 3B). These experiments gave results identical with those shown in Figure 4. Taken together, these observations suggested that procaryotic synthesized α-tubulin was associating nonspecifically, or at best "quasi-specifically", with MTP through the various cycles rather than incorporating into the microtubule lattice.

To address the question of assembly competence and specificity of association, we performed experiments using an alternative assembly/disassembly procedure to remove proteins that were not integral components of the microtubule lattice. This procedure is based on taxol stabilization of microtubules followed by a dissociative treatment with high salt to remove microtubule-associated proteins (Vallee, 1982; Collins & Vallee, 1986; Dinsmore & Sloboda, 1987). In these experiments, procaryotic synthesis reaction crudes were mixed with [^{3}H]Tyr^{451}-tubulin (an internal positive control) and purified by anion-exchange chromatography. The sample was supplemented with MTP and induced to assemble at 37 °C by the addition of GTP. At steady state, the microtubules were stabilized by the addition of taxol and MAPs displaced from the stabilized microtubules by the addition of 0.5 M NaCl. The samples were centrifuged through 50% sucrose cushions and subjected to repetitive cycles of microtubule depolymerization (with calcium) and microtubule assembly (with taxol) (Materials and Methods). Under these stringent assembly conditions, the [^{3}H]tubulin internal control maintained a stable specific activity through the various cycles of assembly/disassembly whereas the specific activity of the procaryotic

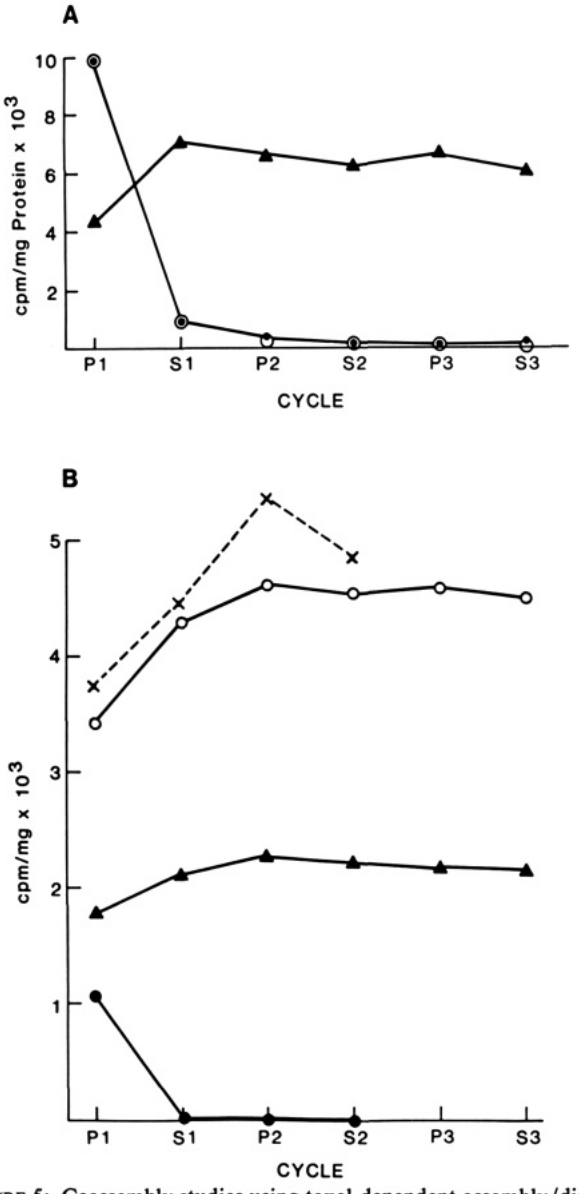

FIGURE 5: Coassembly studies using taxol-dependent assembly/disassembly. (A) ^{35}S-Labeled procaryotic α-tubulin was mixed with ^{3}H-tyrosylated tubulin dimer, purified by anion-exchange chromatography, supplemented with MTP, and carried through three cycles of taxol-dependent assembly/disassembly (Materials and Methods). The ^{35}S (O) and ^{3}H (▲) specific activities of samples removed at pellet (P) or supernatant (S) stages were determined by scintillation counting and protein concentration determinations (Lowry et al., 1951). A parallel control study was done with radiomethylated denatured α-tubulin from bovine brain (●). To facilitate comparisons, specific activities of procaryotic α-tubulin were normalized relative to denatured control. (B) α-Tubulin mRNA transcribed from pGEM3α10 using T7 RNA polymerase was translated in rabbit reticulocyte lysates containing [^{35}S]methionine. Translation products were mixed with ^{3}H-tyrosylated tubulin dimer, purified by anion-exchange HPLC, supplemented with MTP, and carried through three cycles of taxol-dependent assembly/disassembly. Radiomethylated denatured α-tubulin was used as a negative control. This figure shows the supernatant (S) and pellet (P) specific activities [determined as in (A)] as a function of cycle number where (O), (▲), and (●) denote the eucaryotic ^{35}S-α-tubulin, ^{3}H-tyrosylated tubulin, and denatured bovine brain α-tubulin, respectively. Also included is a study (×) in which ^{35}S-α-tubulin from the reticulocyte lysates was incubated one-to-one with the S-30 bacterial extracts to test for the possible presence of factors in the bacterial extracts which inhibit coassembly (see Results).

synthesized ^{35}S-α-tubulin decreased dramatically by the first supernatant and displayed a level of "coassembly" indistinguishable from denatured α-tubulin controls (Figure 5A).

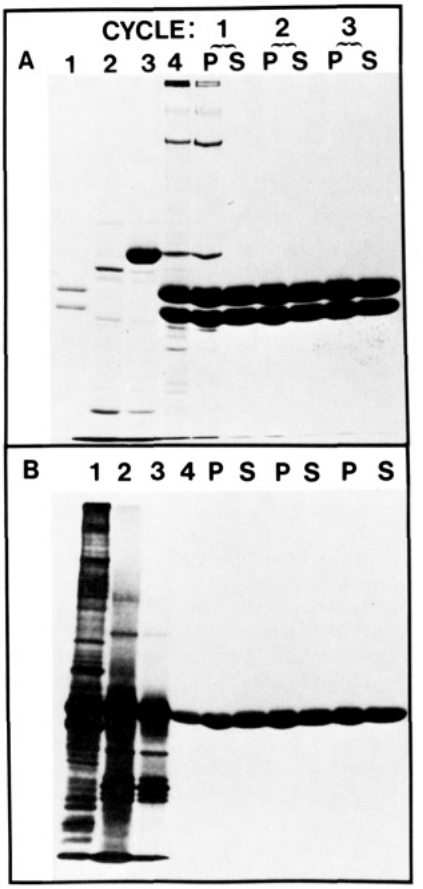

FIGURE 6: Analysis of eucaryotic coassembly studies. Aliquots containing equal amounts of total protein from consecutive cycles of the taxol assembly/disassembly procedure (Figure 5B) were analyzed by SDS–PAGE. The gels were stained with Coomassie Blue-R (A), impregnated with PPO, dried, and fluorographed at −70 °C for 48 h (B). The last seven lanes contain both ^{35}S and ^{3}H radioactivity in ratios of approximately 10:1. Because of the 9-fold greater emission energy of the ^{35}S isotope, ^{35}S radioactivity accounts for ~99% of the fluorogram intensity. Lanes 1–4 denote, respectively, radiomethylated tubulin markers, the crude translation reactions before and after Sephadex G-25 chromatography, and HPLC-purified α-tubulin supplemented with MTP carrier. The intense Coomassie-staining band at ~65 kDa in lane 3 of (A) is BSA. Panel A shows that MAPs (and assembly-incompetent tubulin) are significantly diminished by S1 and essentially absent following P2. The initial increase in specific activity at S1 followed by constant specific activity shown in Figure 5B is borne out in panel B. Note that lane 3 in (B) represents 2 μL of a 100-μL reaction crude while lane 4 corresponds to 0.07-μL "equivalents" of the initially synthesized α-tubulin.

This assay was sufficiently sensitive that if 0.5–1% of the HPLC-purified procaryotic α-tubulin was assembly-competent, it would have been detected.[2]

Similar assembly studies were conducted with α-tubulin produced in the eucaryotic-based translation system. In contrast to the *E. coli* lysates, the eucaryotic-based translation system generated α-tubulin which was assembly-competent by both the temperature-dependent (Figure 4) and taxol-dependent (Figures 5B and 6A,B) assembly/disassembly procedures. When radiolabeled α-tubulin was purified from rabbit reticulocyte lysates by anion-exchange HPLC and supplemented with MTP, it displayed constant specific activity through two cycles of temperature-dependent assembly/dis-

[2] This estimate assumes (i) an ability to measure cpm at a level of twice background (≤25 cpm) with 8-fold losses of protein from P1 to S3 and (ii) that the taxol coassembly procedure fully discriminates between adventitious association with the microtubule and incorporation into the microtubule lattice (Figure 6).

Table I: Taxol-Dependent Coassembly Studies of Eucaryotic α-Tubulin: cpm Recoveries

	total corrected cpm ($\times 10^{-3}$)						
	initial	P1	S1	P2	S2	P3	S3
coassembly 1[a]							
^{35}S	342	271	218	147	88	60	46
^{3}H (×10)	309	280	217	145	86	57	44
mg of protein	35	16	10	6.4	3.9	2.6	2.0
coassembly 2							
^{35}S	602	464	376	239	187	102	82
^{3}H (×7)	637	442	377	238	173	97	82
mg of protein	32	16	11	6.5	5.3	2.9	2.4
coassembly 3							
^{35}S	215	171	130	68	62	–[b]	–
^{3}H (×1.6)	242	147	125	65	63	–	–
mg of protein	23	11	6.0	3.1	3.0	–	–
coassembly 4							
^{35}S	71	47	33	–	–	–	–
^{3}H (×1.8)	74	46	33	–	–	–	–
mg of protein	20	9.2	5.7	–	–	–	–

[a] ^{35}S-Labeled eucaryotic α-tubulin was purified by anion-exchange chromatography. In coassemblies 1 and 2, α-polypeptide was supplemented with [^{3}H]tubulin prior to HPLC purification, whereas in coassemblies 3 and 4 the [^{3}H]tubulin internal standard was added following HPLC purification of the α-polypeptide. The dual-labeled mixtures were supplemented with MTP to the indicated protein mass and carried through one or more taxol-dependent assembly cycles. cpm were corrected for channel leakage using a dual-isotope program on a Beckman LS7500 counter. To facilitate comparisons, ^{3}H cpm were multiplied by the indicated factors (×1.6–×10). [b] Not determined.

assembly (Figure 4). Furthermore, this purified α-tubulin displayed a stable specific activity through three cycles of taxol-dependent assembly/disassembly similar to the behavior of the [^{3}H]tubulin control (Figure 5B). Figure 6A shows that MAPs are significantly diminished by the first supernatant fraction (S1) and essentially absent following the second microtubule pellet (P2). The initial increase in specific activity from P1 to P2 followed by the constant specific activity observed for both the ^{35}S α subunit and the [^{3}H]tubulin control (Figures 5B and 6B) is the result of this removal of MAPs and subsequent enrichment of the sample for tubulin. Consistent with this conclusion, we observed identical coassembly behavior when phosphocellulose-purified tubulin, rather than MTP, was used as carrier protein except that this early rise in specific activity was largely eliminated (data not shown).

In Table I we tabulate total ^{35}S and ^{3}H cpm recovered at each assembly/disassembly cycle for four taxol-based coassembly studies using HPLC-purified α-tubulin translated in the reticulocyte lysates and [^{3}H]tubulin controls. Our data show that the ^{35}S and ^{3}H radiolabels incorporate into microtubules to similar degrees and display similar losses at each cycle stage. This finding demonstrates that the synthesized α subunit isolated by HPLC is *fully* functional by our assembly criterion. In addition, this level of assembly competence was unaffected by whether microtubule protein was added prior to or following HPLC purification (Table I). Since ~40% of the synthesized α-tubulin and the [^{3}H]tubulin control were recovered in the HPLC-purified fraction, we conclude that at least 40%, and perhaps as much as 100%, of the human α-tubulin produced in rabbit reticulocyte lysates is functional.

Properties of the Isolated α-Tubulin Subunit. The studies described above demonstrated that functional α-tubulin, as judged by assembly competence, can be synthesized in eucaryotic cell lysates and isolated independently of β-tubulin. In this section, we characterize the stability of the isolated α subunit relative to the α subunit of the native tubulin heterodimer and probe for possible conformational differences between unassociated and β-tubulin-associated α subunits using limited proteolysis and reductive methylation.

Tubulin heterodimer has a ~4–6-h half-life at 37 °C at neutral pH in 2.5 M glycerol buffer [cf. Sternlicht and Ringel (1979)]. The fact that we were able to recover high levels of assembly-competent α-tubulin from the reticulocyte lysates

Table II: Stability Study of Eucaryotic α-Tubulin[a]

conditions	cpm initial[b]	cpm final[b]	% assembly competence[c]
control			
^{35}S	5000	9440	100
^{3}H	5000	6430	100
4 °C, overnight			
^{35}S	5000	7900	85
^{3}H	5000	7400	115
37 °C, 3 h			
^{35}S	5000	6940	74
^{3}H	5000	4530	71

[a] ^{35}S-Labeled eucaryotic α-tubulin was purified by anion-exchange chromatography, mixed with 0.5 mg/mL BSA, and divided into three equal aliquots: one for the 37 °C incubation, one for the overnight 4 °C incubation, and one for an immediate coassembly study (the control). [^{3}H]Tyr^{451}-tubulin samples (3 mg/mL) were incubated without prior chromatographic purification. At the end of the incubation periods, aliquots containing approximately equal counts of ^{3}H- and ^{35}S-radiolabeled protein were combined, mixed with MTP, and carried through one cycle of taxol-dependent assembly/disassembly. [b] To facilitate comparisons, specific activities at the start of the first cycle, which varied from 3600 to 5600 cpm/mg, were taken as 5000 cpm/mg. The final values at the end of the first cycle (S1) were normalized to this common initial value. [c] Relative to the control samples.

(Table I) suggested that the unassociated α-tubulin subunit was stable. We wished to know whether the unassociated subunit was at least as stable as the α subunit of the tubulin heterodimer. To examine this question, HPLC-purified ^{35}S-α-tubulin and [^{3}H]Tyr^{451}-tubulin preparations were incubated separately in PB+2.5MG buffer at 4 °C overnight or at 37 °C for 3 h. The ^{35}S and ^{3}H samples were then combined, supplemented with MTP carrier, and subjected to one cycle of taxol-dependent coassembly. The renormalized specific activities of the initial and S1 supernatants are shown in Table II along with the values obtained from freshly prepared controls. Both samples retained essentially full assembly competence following overnight incubation at 4 °C and approximately 73% of their assembly competence following a 3-h incubation at 37 °C.

Brown and Erickson (1983) showed that the α subunit in the tubulin heterodimer is highly resistant to chymotrypsin cleavage under limiting conditions whereas the β subunit is cleaved into two fragments of 34 and 19 kDa. In Figure 7, we show limited chymotrypsin digestion patterns of HPLC-

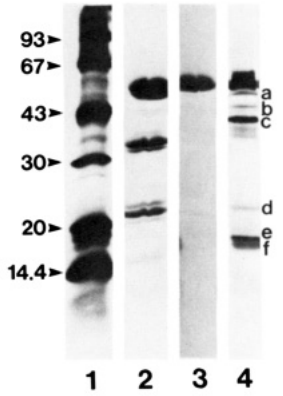

FIGURE 7: Limit chymotryptic studies of α-tubulin. HPLC-purified (monomeric) α-tubulin synthesized in rabbit reticulocyte lysates was supplemented with 1.1 mg/mL BSA or mixed with MTP and carried through one cycle of taxol-dependent assembly (2.9 mg/mL final concentration) to affect incorporation into the heterodimer. 30-μL aliquots were digested with 1.5 μg of chymotrypsin for 25 min at room temperature, electrophoresed on 15% SDS–PAGE, then stained with Coomassie Blue, and autoradiographed. Molecular weight markers are shown in lane 1. Lane 2 shows the Coomassie-stained pattern derived from digests of the taxol coassembly mixture; lane 3, the corresponding autoradiogram; lane 4, the autoradiogram pattern derived from digests of monomeric α-tubulin containing BSA carrier. Bands a–f of lane 4 denote cleavage fragments with molecular weights of ~48K, 44K, 40K, 21K, 18K, and 17K, respectively. The weak unlettered bands immediately below c are "artifacts" of the preparation and were also present in undigested samples. Identical results were observed over a wide range of conditions, e.g., when digestions were done at 37 °C; when phosphocellulose-purified tubulin was substituted for MTP; and when chymotrypsin:protein w/w ratios were varied from 1:20 to 1:250 (data not shown).

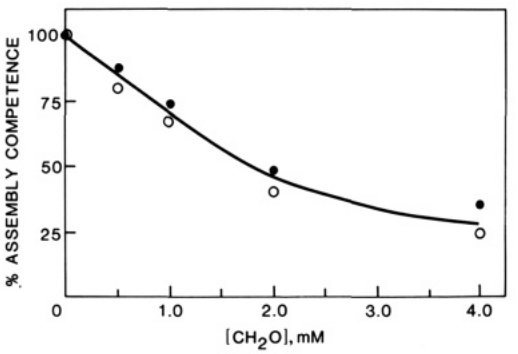

FIGURE 8: Lys-394 region of α-tubulin has a similar conformation in the unassociated and heterodimeric states. ^{35}S-Labeled α-tubulin from reticulocyte lysates was purified by HPLC, supplemented with BSA to a final protein concentration of 1.1 mg/mL, and reacted at 37 °C for 15 min with varying concentrations of HCHO and NaCNBH$_4$ according to Szasz et al. (1986). A parallel methylation control study was done with ^{3}H-tyrosylated tubulin at ca. 3.8 mg/mL. The corresponding ^{35}S- (○) and ^{3}H-methylated (●) samples were combined, supplemented with MTP carrier, and subjected to one cycle of temperature assembly/disassembly. Relative assembly competence as a function of HCHO was estimated by comparing radiolabel retentions at the end of this assembly cycle with that from nonmethylated controls.

purified monomeric ^{35}S-α-tubulin in the presence of BSA carrier (lane 4) or following incorporation into heterodimers after one cycle of taxol-dependent coassembly (lanes 2 and 3). Digestions were analyzed by electrophoresis through 15% SDS gels stained with Coomassie blue (lane 2) and autoradiographed (lanes 3 and 4). Lane 2 shows that under these limited proteolysis conditions tubulin is cleaved into two fragments of ~33 and 22 kDa. Lane 3 shows that these fragments do not contain radiolabel; hence, they must be derived from β-tubulin in agreement with Brown and Erickson (1983). Therefore, this lane shows that once α-tubulin is incorporated into the heterodimer, it is resistant to chymotrypsin cleavage. In contrast, monomeric α-tubulin is readily cleaved into major products of ~40 and 18 kDa and minor products of ~48, 44, 21, and 17 kDa (Figure 7, lane 4). (Two of these minor products migrating at 48 and 44 kDa are also faintly present in lane 3.) These different proteolytic patterns may reflect conformational differences between the unassociated and associated α subunits or may be due to exposure of subunit–subunit contact site(s) that normally is (are) buried in the heterodimer (see Discussion).

To examine whether these differences in chymotrypsin proteolysis reflected large conformational changes in the α subunit upon association with β-tubulin, we used reductive methylation to probe the conformation of the C-terminal domain. In these experiments, ^{35}S-labeled α-tubulin purified by HPLC was methylated in the presence of BSA with limiting concentrations of HCHO and NaCNBH$_3$ for 15 min at 37 °C. A parallel methylation control was done with ^{3}H-tyrosylated tubulin. The HCHO concentration range chosen ($\leq$4 mM) should limit the methylation reaction primarily to the highly reactive amino residues (Sherman et al., 1983), while the concentrations of BSA and ^{3}H-tyrosylated tubulin were adjusted to ensure equal concentrations of lysyl residues in the two reactions. Following methylation, the ^{35}S and ^{3}H samples were combined, supplemented with MTP, and assayed for their assembly competence (Figure 8). ^{35}S-α-Tubulin samples, which were methylated in the unassociated state, were inhibited to the identical extent as the [^{3}H]tubulin samples. We interpret this finding as evidence that the unassociated α subunit has a highly reactive amino group similar to that observed in the heterodimer and tentatively assign this residue to Lys-394 in the C-terminal domain (see Discussion).

N-Terminus Analyses Indicate That Procaryotic α-Tubulin Is N-Formylated. The eucaryotic expression studies demonstrated that the isolated α-tubulin polypeptide synthesized independently of the β subunit is both stable and assembly-competent. The following question then remained: Why did the procaryotic-expressed α-tubulin synthesized in vitro fail to assemble?

We were concerned that the failure of α-tubulin synthesized in the procaryotic system to coassemble into microtubules might reflect the presence of inhibitors in the *E. coli* lysate. To test this possibility, we mixed the reaction crudes from the reticulocyte lysate one-to-one with *E. coli* lysates supplemented with pKK223-3 (the parent expression vector for pKKHαT 11/2 which only expresses β-lactamase). This mixture was incubated for 15 min at 30 °C, HPLC-purified, supplemented with MTP carrier, and then subjected to two cycles of taxol-dependent coassembly. A ^{3}H-tyrosylated tubulin control was carried out in parallel. The ^{35}S mixture coassembled to a similar extent as untreated samples and showed no evidence of assembly inhibition as judged by specific activity profiles (Figure 5B). Furthermore, the percent of initial cpm retained at S1 was identical within experimental error with the retention value obtained for the ^{3}H-tyrosylated tubulin control. Hence, inhibitory factors in the *E. coli* lysate were not a major problem.

To further elucidate the failure of procaryotic α-tubulin to coassemble, we investigated whether the α subunit synthesized in *E. coli* lysates could exchange into tubulin heterodimer, a reaction which very likely is a prerequisite for coassembly (see below and Discussion). When procaryotic α-tubulin crude synthesis reactions were incubated with bovine brain MTP for 45 min at 37 °C and then further incubated with anti-β-tubulin antibody (Blose et al., 1984), we were unable to specifically immunoprecipitate the ^{35}S-α-polypeptide. However, specific

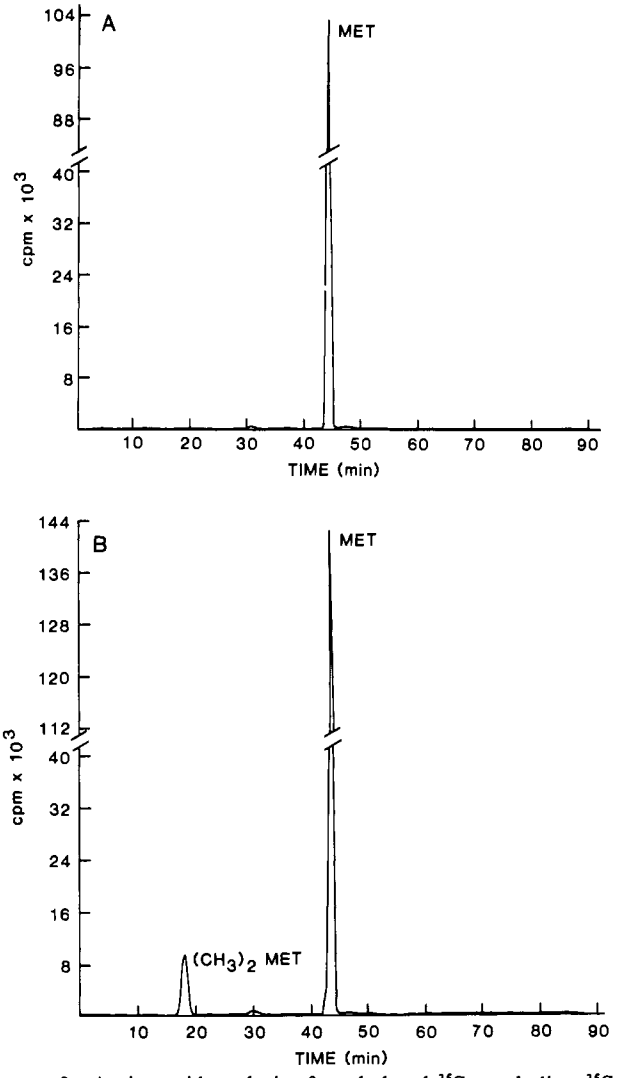

FIGURE 9: Amino acid analysis of methylated ^{35}S-α-tubulin. ^{35}S-Labeled in vitro synthesized procaryotic (180 μL) and eucaryotic (150 μL) reaction products were chromatographed on Sephadex G-25, supplemented with 380 μg of MTP, and the ^{35}S-α-tubulin was isolated by anion-exchange HPLC. Samples were denatured, extensively methylated, hydrolyzed, and analyzed on a Beckman Model 119CL amino acid analyzer (Materials and Methods). Procaryotic α-tubulin hydrolysates contain internal methionines eluting at 44 min but lack a dimethylmethionine peak, indicating that the N-terminal methionine is either blocked or missing (A). In contrast, eucaryotic α-tubulin digests displayed an N,N-dimethylated methionine peak at 18 min with a radiolabel content in the expected 1:9 ratio relative to internal methionines eluting at 44 min (B).

immunoprecipitation was obtained with *eucaryotic-based* ^{35}S-α-tubulin controls following MTP incubation, consistent with heterodimeric exchange in the case of the controls (data not shown). These findings argued that the failure of procaryotic α-tubulin to coassemble occurred at the exchange level or earlier and was most likely caused by a defect(s) in the in vitro synthesized procaryotic α-tubulin. Clearly, incomplete synthesis was not a factor as discussed above. We attempted to verify that the amino-terminal methionine of α-tubulin coded for by the cDNA was intact in these procaryotic lysates. This experiment was considered important because the N-terminal methionines are cleaved from 60% of nascent *E. coli* proteins (Vogt, 1970; Ben-Bassat et al., 1987). When procaryotic radiolabeled α-tubulin was HPLC purified and subjected to N-terminus analysis by reductive methylation (Sherman et al., 1983; Haas & Rosenberry, 1985), no dimethylmethionine residues were detected, suggesting that the N-terminal methionine was either missing or blocked (Figure 9A). In contrast, the N-terminus of the eucaryotic-expressed α-tubulin contained a free, unblocked N-terminal methionine, which could be readily dimethylated (Figure 9B).

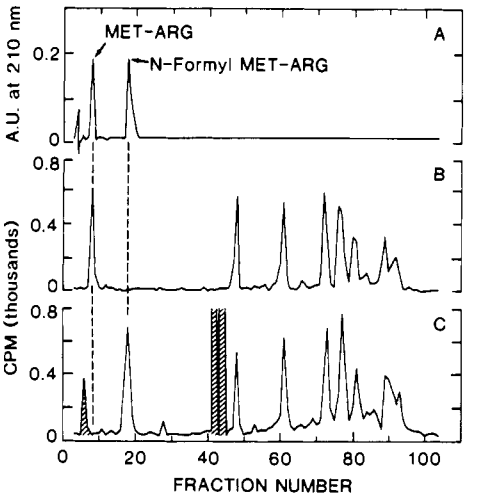

FIGURE 10: Chromatographic analysis of trypsin-digested ^{35}S-α-tubulin. In vitro synthesized ^{35}S-labeled procaryotic and eucaryotic α-tubulins were purified by anion-exchange chromatography, supplemented with MTP, and alkylated with *N*-ethylmaleimide prior to tryptic digestion (Materials and Methods). The digests were chromatographed at room temperature on an analytical Synchropak RP-P C18 column (300-Å pore size). Absorbance was monitored at 210 nm, and 0.5-mL fractions were collected for scintillation counting. (A) Met-Arg and *N*-formyl-Met-Arg dipeptide standards (25 nmol); (B) eucaryotic α-tubulin digests; (C) procaryotic α-tubulin digests. All 10 methionyl-containing fragments predicted from the α-tubulin sequence were detected. Except for the dipeptide fragments, which emerged at early times in (B) and (C), digestion patterns from the two expression systems were very similar. The eucaryotic digests contained a Met-Arg fragment (B) which was absent in the procaryotic digest. The procaryotic digests instead contained an *N*-formyl-Met-Arg fragment (C). Recoveries of Met-Arg and *N*-formyl-Met-Arg were essentially complete and stoichiometric. In this study, significant amounts of contaminating radiolabel (hatched areas) were detected, which were eliminated in later studies by extensive dialysis prior to tryptic digestion.

The nature of this amino-terminal modification was further investigated by tryptic digestion and reverse-phase HPLC chromatographic analysis. On the basis of the cDNA sequence for human α-tubulin (Cowan et al., 1984), trypsin cleavage should give rise to a radiolabeled N-terminal Met-Arg dipeptide. When human α-tubulin synthesized in the procaryotic lysates was analyzed in this manner (Figure 10C), the radiolabeled fragment that was generated coeluted with *N*-formyl-Met-Arg standards (Figure 10A). In contrast, HPLC analysis of tryptic-digested ^{35}S-α-tubulin synthesized in reticulocyte lysates demonstrated a radiolabeled Met-Arg fragment and no *N*-formyl-Met-Arg (Figure 10B), as expected for a free unblocked N-terminal methionine. These results established that the amino-terminal methionine of α-tubulin synthesized in procaryotic cell lysates was N-formylated and that this N-formyl modification was correlated with the loss of assembly competence (see Discussion).

Discussion

In this study, we present the first attempts to characterize the biophysical and biochemical properties of the isolated α subunit of tubulin. We devised a novel strategy using cDNA expression vectors to synthesize α-tubulin in procaryotic and eucaryotic cell lysates, since it has not been possible to isolate the individual α- and β-polypeptides in the native state from preparations of tubulin heterodimers. This approach produced small amounts of soluble, highly radiolabeled α-tubulin which could then be used to assay for assembly competence, stability,

and conformation of the monomeric α subunit. In addition, this method offers the potential for a variety of other experiments to probe more deeply the molecular basis for tubulin function.

We described the construction and use of two expression vectors, pKKHαT 11/2 and pGEM3α10, for the in vitro synthesis of α-tubulin. Both vectors produced full-length human keratinocyte α-tubulin polypeptides on the basis of several criteria, including their immunoreactivity with the monoclonal antibody YL1/2, their elution from an anion-exchange column at salt concentrations similar to that of the tubulin heterodimer (cf. Figures 2 and 3), and their tryptic digestion patterns which revealed 10 methionyl-containing fragments (Figure 10).

pGEM3α10 generated mRNA transcripts which produced assembly-competent α-tubulin when translated in rabbit reticulocyte lysates (Figures 4 and 5B; Table I). Under these synthesis conditions, the α-tubulin polypeptide in the rabbit reticulocyte lysate existed as a monomeric subunit and not as a heterodimer with β-tubulin, as shown by immunoprecipitation studies with an anti-β-tubulin antibody (Figure 2A) and by limiting proteolytic digestion with chymotrypsin (Figure 7). To the best of our knowledge, the pGEM3α10 expression system permitted the first successful in vitro characterization of an isolated native subunit of tubulin (cf. Figures 7 and 8) and its first unambiguous reconstitution into the tubulin heterodimer (Figures 5–7). Although other investigators had successfully *cotranslated* α- and β-tubulin mRNAs from crude or partially purified brain mRNA preparations (Jorgensen & Heywood, 1974; Gozes et al., 1975; Floor et al., 1976; Gilmore-Herbert & Heywood, 1976), only Cleveland et al. (1978) demonstrated assembly competence using temperature-dependent assembly. Figures 4 and 5A demonstrate that even this criterion may not be sufficient to ensure that proteins are true components of the microtubule or the tubulin heterodimer. By using a taxol/Ca^{2+}-based coassembly protocol, we were able to distinguish specific incorporation of a radiolabeled subunit into the microtubule lattice from nonspecific interactions with the microtubule (Figures 4 and 5A; Figure 6).

We showed that the isolated α subunit produced in rabbit reticulocyte lysates is at least as stable as α-tubulin in the heterodimer (Table II). In contrast with our in vitro result, the pulse–chase experiments of Kemphues et al. (1982) appeared to indicate that α-tubulin is unstable when synthesized in the presence of a mutant β subunit in *Drosophila* testes. This instability most likely reflects factors other than the intrinsic stability of the α-polypeptide. We also showed that the monomeric α-tubulin subunit becomes highly resistant to chymotrypsin following coassembly with bovine brain MTP (Figure 7) which we attribute to the formation of the hybrid tubulin heterodimer α(human)β(bovine) [cf. Brown and Erickson (1982)]. Whether this resistance results from a conformational change in α-tubulin upon association with the β subunit or is the result of burying a previously exposed chymotrypsin-sensitive site (i.e., at an intersubunit contact region) is not known.[3] Irrespective of the mechanism accounting for the proteolytic differences, we suspect that the conformation of α-tubulin may not be grossly altered upon heterodimer formation. In experiments using reductive methylation, for example, we observed identical patterns of assembly inhibition for α-tubulin in the unassociated and dimeric states (Figure 8). Since in the dimeric state this inhibition results from modification of a highly reactive lysyl residue, Lys-394, in the C-terminal domain of α-tubulin (Szasz et al., 1986) it seems reasonable to conclude that this lysyl residue is identically reactive in the unassociated subunit. If this interpretation is correct, then the polypeptide conformation in the vicinity of Lys-394 thought to be responsible for its enhanced reactivity [cf. Szasz et al. (1986)] is maintained in the isolated subunit.

We also investigated the in vitro synthesis of α-tubulin in procaryotic lysates using pKKHαT 11/2. This was a natural extension of previous work in which we expressed the isolated α subunit in vivo in *E. coli*. This vector yielded insoluble α-tubulin when expressed in vivo (Yaffe et al., 1986) and soluble but assembly-incompetent α-tubulin when expressed in vitro (Figures 4 and 5A). In contrast with α-tubulin expressed in reticulocyte lysates, the α subunit synthesized in *E. coli* lysates was unable to coassemble by the temperature-dependent and taxol-dependent assembly assays. For example, its displacement from the microtubule during the taxol assembly/disassembly procedure paralleled the behavior of the MAPs (compare Figure 5A and Figure 6A). Because MAPs are positively charged proteins and α-tubulin contains a highly negatively charged C-terminus, it seems probable that procaryotic α-tubulin was associating nonspecifically with the MAPs by electrostatic interactions during the temperature-dependent assay (Figure 4).

Immunoprecipitation studies with DM1B, an anti-β-tubulin antibody (Blose et al., 1982), suggested that procaryotic α-tubulin could not exchange into tubulin dimer. We suspect that this failure is due to formylation of the N-terminal methionine. The first 3 amino acids at the N-termini of both α- and β-tubulin are highly conserved, and *no alterations* in these sequences have been detected in more than 13 α-tubulins and 30 β-tubulins sequenced to date, consistent with a specific structural or functional role for these residues. In fact, studies based on aminomethylation argued that this methionine residue is involved in a salt-bridge interaction in the heterodimer (Sherman et al., 1983; also unpublished data), an interaction that would be prohibited if this residue were N-formylated. Although we cannot completely dismiss alternative explanations, e.g., subtle pH or redox potential differences which might affect procaryotic α-tubulin conformation, we consider these possibilities unlikely. However, if these subtle effects were important, they would have to occur during the initial phases of protein folding since the assembly competence of eucaryotic α-tubulin was unaffected by mixing with *E. coli* lysates (Figure 5B). It is also unlikely that formation of aberrant disulfide bonds was involved, since DTT or β-mercaptoethanol was present in large excess in the synthesis reactions and in the assembly buffers. In addition, the differences between procaryotic- and eucaryotic-synthesized α-tubulin cannot be ascribed to posttranslational modifications since neither system supports such reactions.

Our finding of an *N*-formylmethionine at the amino terminus of α-tubulin synthesized in coupled transcription–translation reactions is in agreement with earlier reports (Capecchi, 1966) that many nascent *E. coli* proteins made in a cell-free system contain *N*-formylmethionine. Retention of the *N*-formyl group following translation initiation in procaryotes has been attributed to the lability of the *E. coli* deformylating enzyme in cell-free extracts (Adams, 1968). To confirm that formylation was indeed responsible for the loss

[3] Chymotryptic digestion of "monomeric" α-tubulin (Figure 9) generates fragments (M_r ~48K, 44K, 40K, 21K, 18K, and 17K) similar to those produced by limited tryptic cleavage (M_r ~51K, 41K, 35K, and 14K) of the subunit in the tubulin heterodimer (Brown & Erickson, 1983). Such similarities may reflect cleavages in a common region(s) highly susceptible to proteases, i.e., between folded domains [cf. Mandelkow et al. (1985)].

of assembly competence and to test whether its effects on the coassembly/exchange of procaryotic α-tubulin with MTP were reversible, we attempted to remove the *N*-formyl group using deformylases isolated from *E. coli* (Fry & Lamborg, 1967) and *Bacillus subtilis* (Takeda & Webster, 1968). To date, these experiments have proven unsuccessful due to the extreme lability of this enzyme and its inherent contamination with proteases in most of our preparations.

Bond et al. (1986) tested the effect of highly divergent primary sequences on microtubule function by transfecting a chicken–yeast chimeric β-tubulin gene into mouse fibroblasts. Their construct contained the first 344 amino acids of the chicken N-terminus, a sequence highly homologous to mouse β-tubulin genes, and the widely divergent 144 amino acids of the yeast β-tubulin C-terminus. These authors found that the chimeric gene product incorporated efficiently into all subclasses of microtubules in vivo. In contrast, we have implicated a single modification at the N-terminal methionine of α-tubulin as the cause of that subunit's loss of assembly competence in vitro. Both observations can be rationalized by the appreciation that, unlike the highly conserved amino termini, the C-termini residues 415–451 essential for microtubule assembly (Maccioni et al., 1986) have undergone significant evolutionary changes (Ponstingl et al., 1981; Toda et al., 1985; Silflow & Youngblom, 1986; Sullivan & Cleveland, 1986). Therefore, these differences between the N- and C-termini are consistent with a model which assigns an essential structural role to the N-terminus and variable or modulatable functional roles to the C-terminus. We expect that work in progress using site-directed mutagenesis will support or refute this hypothesis.

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