# Properties of Microtubules Assembled from Mammalian Tubulin Synthesized in *Escherichia coli*†

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ABSTRACT: When isolated from tissues, the αβ-dimeric protein tubulin consists of multiple isoforms which originate from the expression and subsequent posttranslational modification of multiple polypeptide sequences. Microtubules studied in vitro consist of mixtures of these isoforms. It is therefore not known whether dimers composed of single sequences of α- and β-tubulin can polymerize to form microtubules, or whether posttranslational modifications may be necessary for microtubule assembly. To initiate investigation of these questions, rabbit reticulocyte lysate, which contains the cytoplasmic chaperonin CCT and its cofactors, was employed to prepare substantial quantities (tens of micrograms) of active tubulin by in vitro folding of mouse α- and β-tubulins recombinantly synthesized in *E. coli*. This recombinant tubulin is composed of only a single α-chain and a single β-chain. When analyzed after folding by isoelectric focusing, each chain yielded only one band, indicating that neither was detectably posttranslationally modified in the course of the folding reaction. When subjected to assembly-promoting conditions, this tubulin formed microtubules without the addition of any exogenous protein. Electron microscopy showed them to be of normal morphology. Analysis of their protein composition showed that they are composed nearly entirely of recombinant tubulin. These results demonstrate that the naturally occurring mixtures of isoforms are not strictly required for the formation of microtubules. They also open a route to other studies, both biomedical and structural, of fully defined tubulin in vitro.

The microtubules of eukaryotic cells play important roles in intracellular organization and transport, and in mitosis. They are formed by spontaneous polymerization of their fundamental subunit, tubulin, an αβ-dimer whose structure has recently been determined at high resolution (*7, 8*). Their properties are central to many cancer therapeutic strategies (*9*−*11*). Functional study of these self-assembling structures in vitro has been carried out chiefly with tubulin isolated biochemically from mammalian tissue, most often brain. This tubulin is heterogeneous after isolation, consisting, in the case of mammalian brain, of more than 24 isoforms (*12*−*14*). The isoforms arise both from sequence differences [i.e., different *isotypes*, classified by Sullivan (*1*)] and from posttranslational modifications (e.g., polyglutamylation, tyrosination/detyrosination, acetylation, and polyglycylation) of those sequences [see reviews (*15, 16*)]. Despite the growing number of demonstrated structural and functional differences between the isoforms (*5, 17*−*27*), their biological significance is still largely unknown [reviewed by Ludueña (*28*)], although their importance is not in question. These tubulin isoforms are not easily separated while native, and current approaches to understanding their functions necessarily involve partial purification of native dimers by subtractive immunoaffinity chromatography (*29, 30*), a technique which produces incomplete separation of isoforms (e.g., a single type of β-chain is present in mixed dimers with all the isoforms of the α-chains). A potentially fruitful route to biochemical understanding of this problem lies through the assembly of microtubules from single-sequence α- and β- tubulins, native and not yet posttranslationally modified. Although single-sequence tubulins have previously been produced and folded into apparently native conformations, they have not been shown to form microtubules, nor has their pattern of posttranslational modification been investigated.

The repeated experience of many laboratories has shown that unfolded tubulin, whether obtained by biochemical isolation from tissue or by expression in bacteria, cannot be folded or refolded by the routes ordinarily employed with other proteins (e.g., rapid dilution into nondenaturing buffers, slow dialysis, use of detergents). Instead, inactive protein is obtained, often extensively aggregated. This fact has impeded the application of molecular biological methods to functional study of tubulin and microtubules in vitro. In vivo, tubulin polypeptide chains use, and appear to require for their correct folding, a molecular chaperone system consisting of the cytoplasmic chaperonin CCT[1] (also called TRiC or TCP-1 chaperonin, or cytoplasmic chaperonin) and several cofactors.

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[1] Abbreviations: Buffer A, 0.02 M Tris, pH 7.5, 10 mM dithiothreitol, 7.5 M urea; Buffer B, 0.1 M MES, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 0.1 mM GTP, 10% glycerol, pH 6.5; Buffer C, 0.1 M MES, 1 mM DTT, 1 mM EGTA, 4 M glycerol, pH 6.8; CCT, chaperonin containing TCP-1 polypeptide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GMPCPP, guanylyl-(α,β)-methylenediphosphonate; IEF, isoelectric focusing; MAP, microtubule-associated

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An extensive series of studies has established the composition and the structure of the chaperonin (*31*−*38*), as well as the identities and functions of the cofactors [*39*−*45*; see reviews (*46*−*48*)].

Rabbit reticulocyte lysate (RRL) has been shown to contain all of the components of the CCT chaperone system. By tracing the fate of tubulin made by translation of mRNA added to RRL, Yaffe and Sternlicht showed that RRL can fold tubulin into a compact form that is active, in the limited sense that it can be *co-incorporated* with excess biochemically isolated native tubulin into microtubules (*31*−*34*). Cowan and others showed that RRL can also correctly fold denatured tubulin chains when they are rapidly diluted into it from solution in urea (*49, 50*), and it has also been demonstrated that a mixture of denatured α- and *β*-chains, when folded together in RRL, forms α*β*-dimers (*51, 52*). In each of these series of experiments, though, because only tiny amounts (nanograms) of newly folded protein were produced, it was necessary to add a huge excess (>1000-fold) of native tubulin to the solution in order to assemble microtubules. Thus, only co-assembly has been observed so far, and it has not been possible to know whether the newly folded tubulin can make microtubules by itself, or whether the added carrier "recruited" it into them. No investigation of whether the folding process involves posttranslational modifications (either causing them or requiring them) has appeared.

This paper reports the preparation of usable quantities (tens of micrograms) of tubulin dimer by in vitro folding of known single sequences of mouse α- and *β*-tubulin. Fundamental properties of this protein were investigated. First, it was found to form microtubules without addition of "carrier" tubulin. Second, the tubulin composing these microtubules yielded only two IEF bands, one α-isoform and one *β*-isoform, indicating that it had not been detectably posttranslationally modified during the folding process. These results are interpreted to mean that, although the multiple polypeptide sequences and posttranslational modifications present in vivo are doubtless biologically important, neither is essential for the formation of microtubules. They also open a potential route to the functional study of well-defined tubulin in vitro.

## EXPERIMENTAL PROCEDURES

*Materials.* Bovine tubulin was prepared by the method of Williams and Lee (*53*) and stored at −80 °C until use. The nucleotide analogue GMPCPP [guanylyl-(α,*β*)-methylenediphosphonate] was synthesized according to (*54*), as modified (*55*). Reticulocyte-rich rabbit blood (100 mL) was purchased from Pel-Freez (Rogers, AR). Urea was Boehringer-Mannheim 1685−902, which exhibited a relatively low electrical conductivity in solution, indicative of minor contamination by cyanate ion. Urea-containing buffers were prepared frequently and kept at 4 °C to minimize formation of further cyanate ion (*56*). Labeled colchicine, [ring C methoxy-$^3$H], 76.5 Ci/mmol, was obtained from New England Nuclear and diluted with unlabeled colchicine (Sigma, St. Louis, MO) to make a stock of specific activity $1.6 \times 10^{16}$ dpm/mol and concentration 320 $\mu$M (determined from $A_{353}$ and $\epsilon_{353} = 15\,950$).

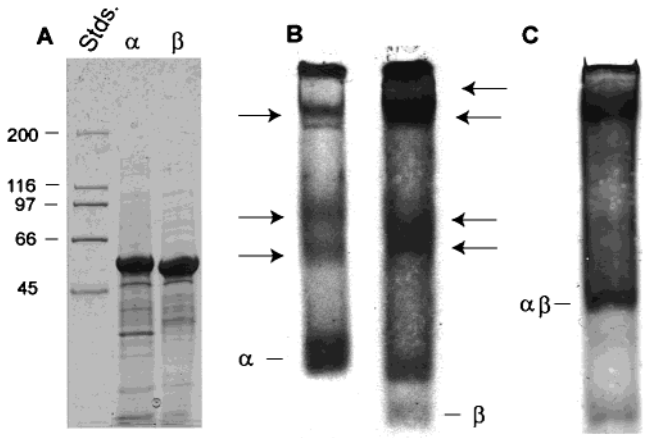


FIGURE 1: Production of polymerizable tubulin: its expression and folding. (A) Coomassie-stained SDS gel (4−15%) of 0.5 $\mu$L of each of the recombinant tubulin solutions. After being concentrated, these solutions were added to the folding-mixture. (B) Nondenaturing gel electrophoresis of aliquots of folding-mixtures containing the same amount of either α- or *β*-tubulin alone (each labeled with [$^{35}$S]tubulin to the same specific activity) was performed and the gel autoradiographed on film. Arrows point to bands which have previously been shown to correspond to complexes of tubulin with the chaperonin and with cofactors [cf. (*86, 87*) and references cited therein]. Note that when equal amounts of protein are added to the reaction mixture, more α-tubulin is folded than *β*-tubulin. (C) A folding reaction carried out at high tubulin concentration with a 2.5:1 ratio of *β*- to α-tubulin and subjected to nondenaturing electrophoresis and autoradiography. A prominent band apparently corresponds to the α*β*-dimer (*51, 52*).

*Protein Expression.* As described in Melki et al. (*37*), tubulin chains [mouse sequences Mα2 and M*β*5 (*2, 57*)] were overexpressed in milligram quantities from pET11 vectors in *E. coli* BL21(DE3), isolated as inclusion bodies from sonicated cells, dissolved in 0.02 M Tris, pH 7.5, 10 mM dithiothreitol, 7.5 M urea (Buffer A), concentrated to about 35 mg/mL by the use of a centrifugal ultrafilter (Ultrafree-30, Millipore Corp.), and stored at −80 °C until use. [$^{35}$S]-Tubulins were produced by addition of labeled cysteine and methionine to defined media. Figure 1A shows a typical preparation.

*Folding of Tubulin.* RRL was prepared by standard methods (*58*) except that protease inhibitors ("Mini Complete, EDTA Free", Boehringer, Mannheim, Germany) were added at the time of lysis. It was stored at −80 °C. *Folding-mixture* was prepared by thawing RRL (10 mL), centrifuging it at 424000*g* and 4 °C for 10 min in a Beckman TL 100.3 rotor to pellet small amounts of inhibitory material, and adding 1/9 volume of 0.4 M MES, 0.1 M KCl, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM DTT, 5 mM ATP, 5 mM GTP, 0.5 M trimethylamine oxide (TMAO), pH 6.8. TMAO partially counteracts the effects of urea in a number of proteins (*59, 60*) and was found in preliminary experiments (not shown) to confer substantial protection against the unfolding of native tubulin by urea. Its inclusion in the folding-mixture increased the yield of folded recombinant tubulin, probably by protecting it, or components of the chaperone machinery, against denaturation.

*Assay of Folding.* Compactly folded tubulin was detected in folding-mixtures by means of nondenaturing electrophoresis on 4.5% gels as described by Gao et al. (*61*) and by

protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; PM buffer, 0.1 M Pipes, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithioerythritol, 1 mM GTP, pH 6.9; RRL, rabbit reticulocyte lysate; TMAO, trimethylamine oxide.

Cowan (*62*). [$^{35}$S]Tubulin was detected in these gels by autoradiography on photographic film or by use of a Phosphorimager (Molecular Dynamics model 445 SI). Folded tubulin chains formed a fast-migrating band (Figure 1B) that could be distinguished from slower-moving complexes of tubulin and from components of the folding machinery. When the mass of folded tubulin was to be measured, the integrated intensity of the fast-moving band was obtained from the Phosphorimager. The instrument was calibrated by measuring the integrated intensity of known amounts of [$^{35}$S]-tubulins incorporated into the same gel.

*Large-Scale Preparation of Tubulin Dimers*. A mixture of recombinant tubulins in ratio $\alpha$:$\beta$ = 1:2.5, at a concentration near 35 mg/mL in Buffer A, and in a volume small enough to keep the final urea concentration below 0.4 M, was added to 10 mL of folding-mixture with rapid stirring. The solution was incubated for 60 min at 30 °C, during which time a band attributable to folded $\alpha\beta$-dimers became evident in nondenaturing electrophoresis (Figure 1C). A tandem arrangement of chromatographic columns, consisting of a 1 × 30 cm column of SP-Sepharose (Amersham−Pharmacia) the outlet of which was connected to the inlet of a 2.5 × 10 cm column of Q-Sepharose, was equilibrated with Buffer B (0.1 M MES, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 0.1 mM GTP, 10% glycerol, pH 6.5) + 0.1 M NaCl. The folding-mixture was applied at 24 °C, and run into the columns at a flow rate of 3−4 mL/min. Flow was continued until hemoglobin began to emerge from the SP column. The columns were then disconnected, and the Q-Sepharose column (which had the folded tubulin bound to it) was moved to 4 °C and washed at 2 mL/min with Buffer B + 0.2 M NaCl until the major peak of unbound protein emerged (about 30 min). A 120 mL salt gradient between Buffer B + 0.2 M NaCl and Buffer B + 0.6 M NaCl was then applied at 2 mL/min. Eluted fractions containing $^{35}$S-labeled tracer tubulin were detected by scintillation counting, concentrated about 250-fold by means of Ultrafree-30 centrifugal concentrators (Millipore), quickly frozen in liquid $N_2$, and stored at −80 °C until use.

*Assembly of Microtubules*. To form microtubules, the concentrated recombinant tubulin solution (approximately 150 $\mu$L) was thawed and centrifuged at 386000*g* for 8 min at 4 °C to remove aggregated protein. To reduce the critical concentration for microtubule assembly to the vicinity of 50 $\mu$g/mL (data not shown), the protein was equilibrated with the nucleotide analogue GMPCPP as follows. Solution was first freed of most exchangeable nucleotide by rapid gel-filtration (centrifugal "Micro Bio-Spin" column Bio-Rad Laboratories, Richmond, CA) into 0.1 M MES, 1 mM DTT, 1 mM EGTA, 4 M glycerol, pH 6.8 (Buffer C), with no added nucleotide or $Mg^{2+}$. Then $MgCl_2$ and GMPCPP were immediately added, to a final concentration of 1 mM each. The resulting solution of tubulin with GMPCPP at its exchangeable site was incubated at 34 °C for 60 min to allow microtubules to form. They were pelleted by centrifugation at 24000*g* for 15 min at 34 °C in a Beckman TL100 rotor. The pellet was resuspended for the analyses shown in Figures 4−6.

*Analysis of Microtubules and Proteins from Microtubules.* Protein concentrations were measured by the method of Bradford (*63*) as modified by Zor (*64*). Electron microscopy was performed on 5 $\mu$L aliquots of solutions applied to freshly glowed grids and negatively stained with 0.5% uranyl acetate. Magnification of the microscope was calibrated by the use of paramyosin paracrystals, and dimensions of objects were measured directly from the photographic negatives by means of a Nikon microcomparator. Isoelectric focusing (IEF) was carried out as described (*14*) in precast Immobiline DryStrip gel strips (pH 4−7) by means of an IPGphor apparatus and its associated strip holders (Amersham Pharmacia Biotech, Piscataway, NJ), in a running solution (*65*) composed of 7 M urea, 2 M thiourea, 20 mM DTT, 4% CHAPS, 0.5% Triton X-100, and 0.5% IPG Buffer, pH 4−7 (Amersham Pharmacia Biotech). To minimize formation of cyanate ion, the urea/thiourea mixture was made fresh each week, and to avoid possible difficulties due to oxidation, DTT was added just before the beginning of the experiment.

The colchicine-binding capacity of RRL was assayed by the use of [$^3$H]colchicine. Protein solutions in PM buffer (60 $\mu$L) were incubated at 37 °C for 3−5 h in the presence of 10 $\mu$M [$^3$H]colchicine and then subjected to gel-filtration on a 0.5 × 5 cm column of Sephadex G-25 Fine (Pharmacia, Piscataway, NJ). The entire protein-containing fraction, separated from the unbound colchicine, was collected and counted in a scintillation counter to determine the amount of colchicine bound. Because binding of colchicine at the very small protein concentrations employed (0.2−0.5 $\mu$M) can be incomplete due to slow kinetics and a finite equilibrium constant (*66, 67*), the colchicine-binding capacity of concentrated RRL was determined by comparison to identically treated control solutions containing bovine tubulin in the same concentration range.

Immunoblots of SDS gels, on a PVDF membrane, were developed by use of alkaline phosphatase, or by chemiluminescent assay (Immun-Star, Bio-Rad Laboratories, Richmond, CA). Immunoblotting of IEF strips was carried out as follows. After focusing, the plastic-backed strips were soaked 20 min in 125 mM Tris, 5% 2-mercaptoethanol, 1% sodium dodecyl sulfate, pH 6.8 (*68*), and then placed face-down against a sheet of PVDF backed by a 2-cm-thick pad of blotting paper. A filter-paper wick, the same length as the IEF strips and several centimeters wide, was brought into apposition with each long edge of each IEF strip. The assembly was covered with a weighted (300 g) glass plate of the same length as the IEF strip, but narrow enough (2 cm) that the wicks projected on each side. The assembly was left for 24 h. Every few hours, the wicks were freshly saturated with buffer. Capillarity gradually drew part of it through the IEF strip and into the blotting paper, producing excellent transfer of the proteins. The resulting blot was probed with primary antibody and developed by chemiluminescent assay as above.

In estimating amounts of tubulin by quantitative immunoblotting, where insensitivity to differences in isotype composition was desired, a combination of TU-01 and Tu-27B was employed. TU-01 (*69*), a monoclonal antibody directed against the N-terminal structural domain of $\alpha$-tubulin, was obtained from Zymed Laboratories, South San Francisco, CA. Although this well-characterized antibody is quite specific for $\alpha$-tubulins (*70*), it reacts quite generally with different isotypes of $\alpha$-tubulin and with $\alpha$-tubulins from disparate species (*71−73*). Tu-27B, the kind gift of Lester Binder, is directed against a strongly conserved epitope in $\beta$-tubulin and reacts with numerous isotypes (*74−77*).

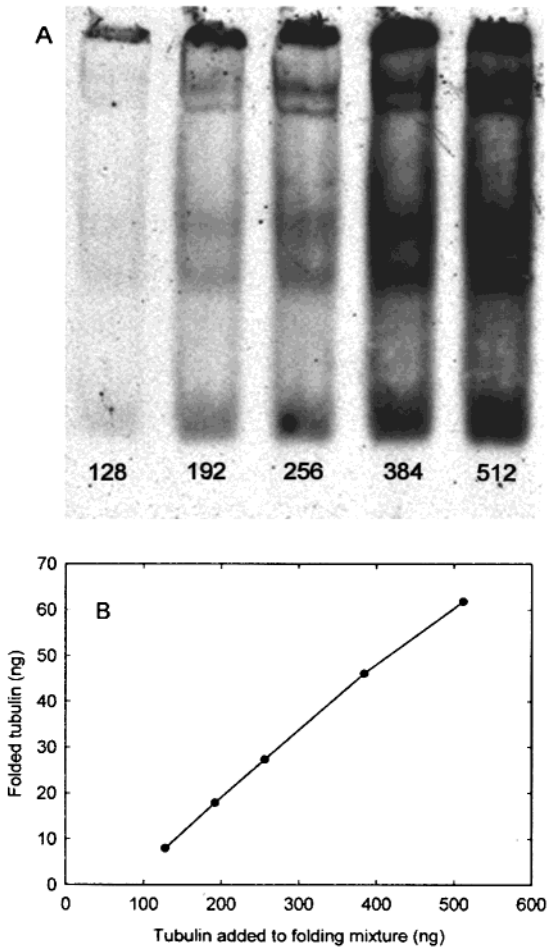


FIGURE 2: Yield of folded tubulin. (A) The indicated number of nanograms of [$^{35}$S]-α-tubulin was added, in a constant volume of Buffer A, to 20 $\mu$L folding-mixtures, which were incubated as described under Experimental Procedures, resolved by nondenaturing gel electrophoresis, and then visualized by means of a Phosphorimager. (B) The amount of folded tubulin, measured by integrating the density of the fast-moving band in (A), is shown as a function of the amount added to the folding-mixture.

## RESULTS

*Yield of Folded Protein.* When folding was initiated by rapidly diluting a urea-containing solution of *E. coli*-derived tubulin into a solution of RRL augmented with an ATP- and GTP-containing buffer (i.e., the folding-mixture), the bulk of the tubulin rapidly precipitated into insoluble aggregates. Some, however, was bound by components of the folding system, folded, and released. To learn how this amount varied with the amount of tubulin added to a constant volume of folding-mixture, a series of folding reactions was carried out in which tubulin-containing solutions, all in the same volume of Buffer A, were added to identical volumes of folding-mixture. Figure 2 shows, for the case of α-tubulin, the observed dependence of the amount of folded tubulin on the amount added. The yield clearly increased steadily with the amount added, and about 10% of the added unfolded tubulin subsequently appeared as compactly folded protein. The capacity of the system did not appear to be saturated at the highest amount examined. In subsequent large-scale experiments, therefore, the largest feasible amount of tubulin was added to the folding-mixture.

*In Vitro Folding and Isolation of Native Tubulin Dimer.* Further assay by nondenaturing electrophoresis of the quantity of folded protein showed that α-chains were folded more efficiently than $\beta$-chains (this phenomenon is visible in Figure 1B). To prepare tubulin dimers efficiently, $\beta$-chains were added to the folding-mixture in greater quantity than α-chains. When both α-tubulin and $\beta$-tubulin were present in the same solution, a new electrophoretic band appeared (Figure 1C). By comparison with previous results (*51, 52*), the presence of this band was tentatively attributed to formation of α$\beta$-dimers. After incubation, subjecting the folding-mixture to rapid isocratic chromatography on a cation-exchange column (SP-Sepharose) was found to remove about 80% of the proteins from the folding-mixture while allowing the folded tubulin, as well as some unfolded tubulin and numerous other proteins, to elute nearly unretarded. As shown in Figure 3, application of the tubulin-containing eluate to an anion-exchange column (Q-Sepharose), followed by elution in a salt gradient at 4 °C, effected a further separation of the remaining components of the folding-mixture. The presence of tubulin, which emerged at the leading edge of a major protein peak, was detected by the inclusion of a small amount of $^{35}$S-labeled protein, and the fractions containing it were pooled and concentrated 250-fold and are called "recombinant tubulin solution". This solution, although greatly enriched in tubulin, is still quite impure (cf. Figure 5A, below). But microtubules can be assembled from it.

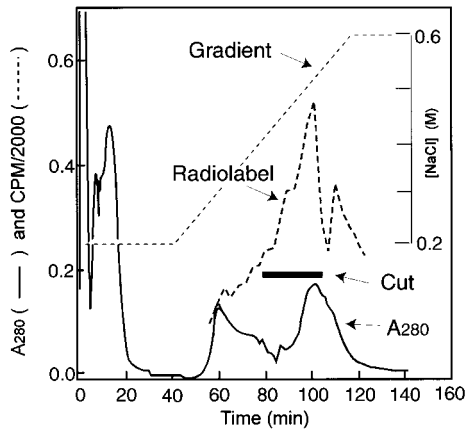


FIGURE 3: Isolation of tubulin from the folding-mixture. A large-scale (10 mL) folding reaction was carried out with a trace amount of [$^{35}$S]tubulin added to a large amount of nonradioactive tubulin. Extraneous proteins were removed by passage through a SP-Sepharose column as described under Experimental Procedures. Subsequent gradient chromatography on Q-Sepharose is shown here. Absorbance (solid line), radioactivity (dashed line), and salt concentration in the eluate (dotted line) were measured, and those tubulin-containing fractions indicated by the horizontal bar were pooled and concentrated.

*Assembly of Microtubules.* To reduce the concentration required for assembly of microtubules, the nucleotide analogue GMPCPP [guanylyl-(α,$\beta$)-methylene-diphosphonate] was first substituted for the GTP presumably present at the exchangeable site of the tubulin in the recombinant tubulin solution (see Experimental Procedures). The solution was then warmed to 34 °C for 60 min to allow assembly to occur, the solution was centrifuged, and the pellet was resuspended in Buffer C augmented with 1 mM $MgCl_2$ and 1 mM GMPCPP. Electron microscopy revealed large numbers of unmistakable well-formed microtubules in this solution. Figure 4 shows representative images of these structures. Measurement of their mean apparent diameter and

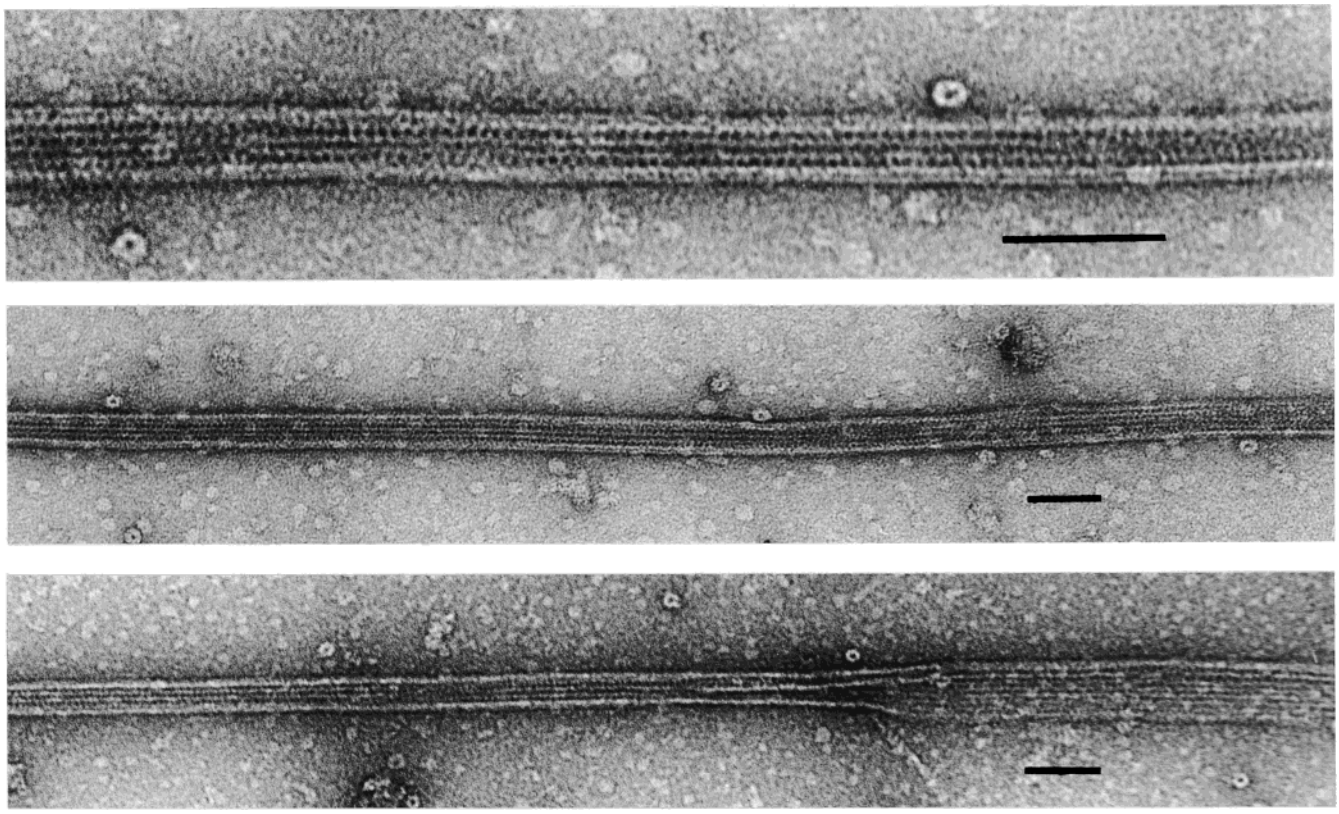

FIGURE 4: Microtubules assembled from recombinant (M$\alpha$2)−(M$\beta$5) tubulin, expressed in *E. coli* and folded in vitro. Note the characteristic substructure in these representative images of negatively stained preparations. The mean apparent diameter of the microtubules was 23.4 nm. The mean apparent spacing between subunits was 3.9 nm. In the bottom image, note the transition from tube to sheet, characteristic of negatively stained microtubules prepared from tubulin free of microtubule-associated proteins. Scale bars: 50 nm.

mean apparent intersubunit spacing yielded values of 29.4 and 3.9 nm, respectively, in good agreement with established values for negatively stained specimens. The striated sheet-like appearance of many of their ends is also typical of negatively stained specimens of microtubules formed from MAP-free tubulin. When either colchicine (10 $\mu$M) or GDP (1 mM) was added to the recombinant tubulin solution prior to the 34 °C incubation, no microtubule-like structures could be observed in the electron microscope. Examination of the resuspended pellet in the light microscope, by means of video-enhanced differential-interference contrast optics (*78*), revealed numerous short linear structures, indistinguishable in appearance from control microtubules assembled from bovine tubulin previously equilibrated with GMPCPP.

*Protein Composition of the Microtubules.* Measurement of the protein content of the centrifugally pelleted microtubules in one preparation yielded 27 $\mu$g, and electrophoretic analysis (Figure 5A) showed them to be composed almost exclusively of tubulin. The assembly step has therefore served to separate tubulin selectively from the many other proteins present in the recombinant tubulin solution, which remained in the supernatant (Figure 5A, right lane). Because RRL may contain small amounts of rabbit tubulin, it is necessary to control for the possibility that the microtubules were formed by the unintended isolation and polymerization of this material. In a first control experiment, two folding-mixtures, of which one contained added tubulin while the other contained only an equal volume of added Buffer A, were carried through the entire procedure (i.e., incubation at 30 °C, chromatography, concentration, substitution of GMPCPP, incubation at 34 °C, centrifugation), and both pellets (although the control pellet was not visible) were subjected to electron microscopy, SDS-gel electrophoresis, and immunoblotting. No microtubules could be visualized electron microscopically in the pellet from the folding reaction to which tubulin had not been added. As shown in Figure 5B, a substantial tubulin band appeared on the silver-stained gel in the lane corresponding to the folding reaction to which tubulin had been added (labeled "Recombinant microtubule pellet"). In the lane corresponding to the folding reaction to which tubulin had not been added (labeled "RRL control pellet"), fainter protein bands were visible in the region of apparent molecular weight where tubulin would appear. The companion immunoblot, probed with an antibody which detects a broad range of $\beta$-tubulins, yielded a strong band from the recombinant microtubule pellet and from bovine tubulin, but none from the control pellet, suggesting that the faint bands do not correspond to tubulin. These results show, first, that detectable microtubules are formed, and appreciable amounts of polymerizable tubulin obtained, only when recombinant tubulin is added to the folding-mixture. Second, they imply that the microtubules are *not* chiefly composed of whatever residual native rabbit tubulin might be present in the RRL. To reveal its composition in more detail, the protein making up the microtubules was analyzed, together with controls, by isoelectric focusing (IEF), with the results shown in Figure 5C. The many (more than 24) isoforms of bovine brain tubulin, detectable by their different isoelectric points (*14*, *28*, *71*, *79*), are clearly visible in the lower strip, which is included as a control. The microtubules made from recombinant tubulin (upper strip), in contrast, contained only two detectable isoforms, at

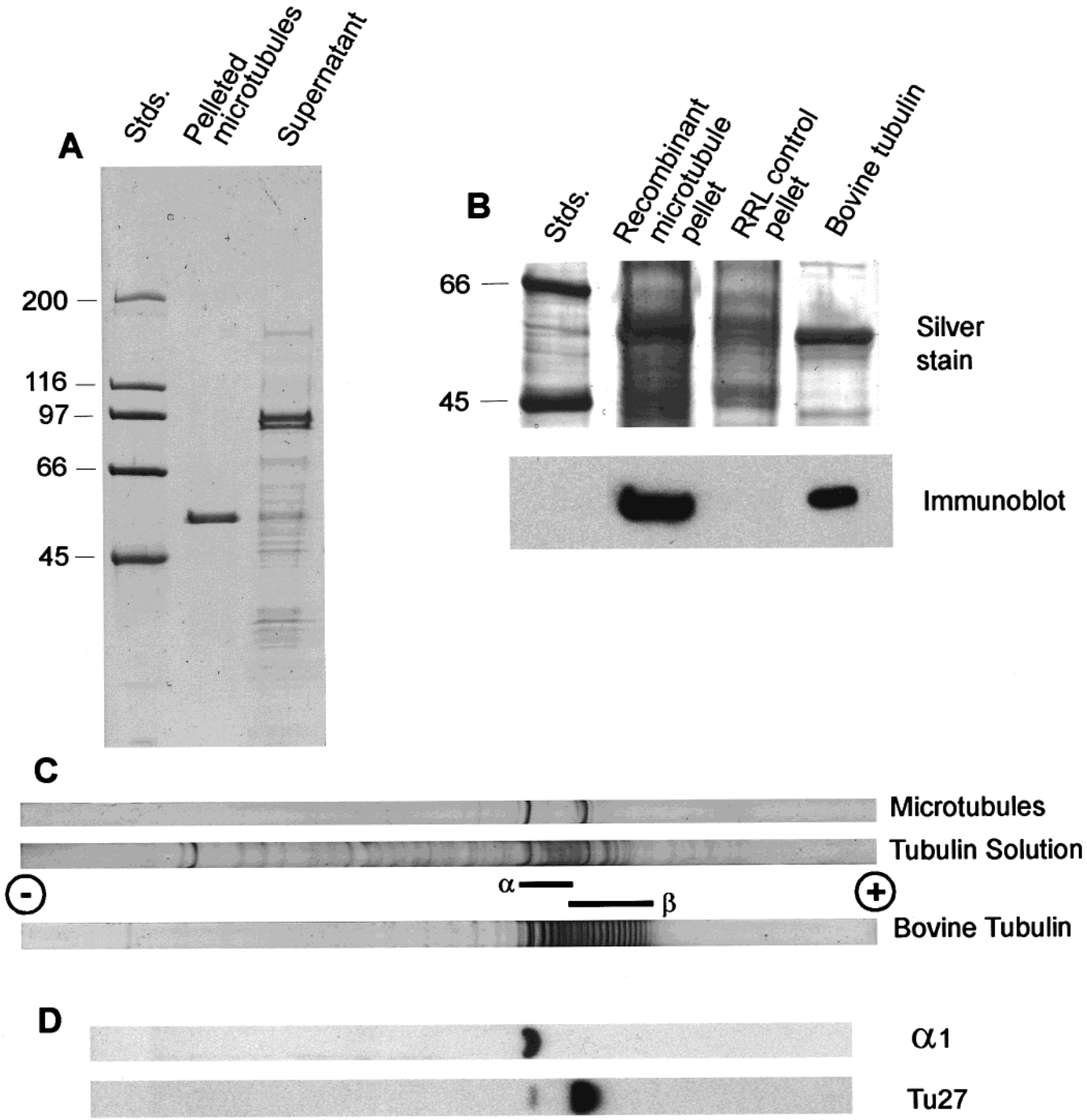


FIGURE 5: Analysis of protein composition of the microtubules. (A) Coomassie blue stained SDS gel of centrifugally pelleted microtubules such as those shown in Figure 4, and of the supernatant from which they were polymerized. Note that they consist almost entirely of tubulin. As is the case when microtubules are assembled from native tubulin in tissue extracts, substantial amounts of other unidentified proteins remained behind in the supernatant. (B) Control to assess the possible presence of tubulin from rabbit reticulocytes. A silver-stained gel (top) and an immunoblot of an identical gel run at the same time and probed with Tu-27B (bottom) show identical aliquots (20%) of a resuspended microtubule pellet and an equivalent aliquot of a control pellet prepared from a "blank" folding reaction to which no tubulin was added. Although the control pellet contains some protein in the molecular mass region near 50 kDa, it does not react with the antibody. Bovine tubulin (1 $\mu$g) was included in the gel for reference. (C) Isoelectric focusing (*14*) analysis of the tubulin in the microtubules (top), of the mixture of α- and $\beta$-chains initially added to the folding-mixture (middle), and of bovine brain tubulin (bottom). The three strips have been horizontally aligned in the figure; the p*I* of a given band thus corresponds closely to its horizontal position. Comparison with the controls shows that only one detectable α-isoform and one detectable $\beta$-isoform are present in the recombinant microtubules and that the isoelectric point of each corresponds to a major band in the bacterial lysate added to the folding-mixture. (The bacterial lysate contains numerous non-tubulin proteins, probably corresponding to the bacterial proteins also visible in Figure 1A.) Gradient of pH from 4 to 7; loads (top to bottom): 2 $\mu$g, 4 $\mu$g, 10 $\mu$g. Strips were stained with Acid Violet 17. Horizontal bars indicate the regions where α-tubulin and $\beta$-tubulin are known to focus (*71, 79*). (D) Immunoblots of two identical IEF gels, each loaded with approximately 4 $\mu$g of protein from recombinant microtubules. One was probed with the α-tubulin antibody α1 (the kind gift of Anthony Frankfurter) and the other with the $\beta$-tubulin antibody Tu27. A small amount of α1 was added to the primary antibody solution in order to mark the position of the α-chain in the strip marked Tu27.

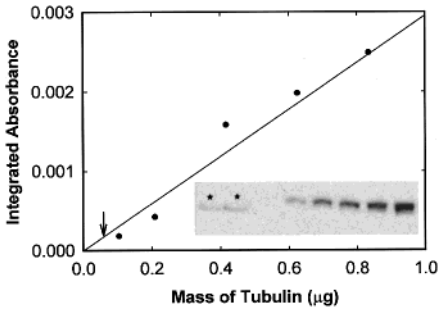


FIGURE 6: Quantitation by immunoblotting of rabbit tubulin present in RRL. RRL (5 mL) was depleted of hemoglobin and concentrated as in the folding reaction (see Experimental Procedures). An aliquot of this concentrated material (6% of the total, corresponding to 295 $\mu$L of RRL) was electrophoresed, together with several lanes containing known amounts of bovine tubulin. This gel (inset) was immunoblotted with a mixture of antibodies to α- and $\beta$-tubulin (Tu-01 and Tu-27B) and detected by chemiluminescent assay. The integrated intensities of the bovine tubulin were used as standards to quantitate the tubulin present in RRL, as shown in the graph. The vertical arrow indicates the integrated density obtained from the RRL-containing lanes (indicated by * * in the inset). The visible mobility difference between the RRL bands and the standards may correspond to a similar difference noted by Murphy et al. (*88*).

positions corresponding visually to those expected from the most negatively charged variants of the α- and $\beta$-chain isoforms (*71*). The isoelectric pHs of these bands coincided with those of the major bands visible in the starting mixture of α- and $\beta$-chains (middle strip). Immunoblotting of the two tubulin bands from the microtubules (Figure 5D) confirmed their identification as α- and $\beta$- chains. These observations are interpreted to mean that the single α- and $\beta$-tubulin sequences expressed in *E. coli* were folded and incorporated into microtubules intact and without detectable posttranslational modification.

Although the data of Figure 5 show the microtubules to be composed largely of the recombinantly produced mouse α- and $\beta$-tubulin sequences, a further test seemed useful to assess the possible presence in them of small amounts of other tubulins. The reason is that although reticulocytes have long been known to be nearly lacking in microtubules (*80, 81*), they do contain detectable amounts of rabbit tubulin, some of which might copolymerize with the mouse tubulin. We therefore estimated the amount of tubulin present in RRL by two approaches. Aliquots (10 mL) of RRL were passed over SP-Sepharose exactly as in the tubulin-folding procedure to remove hemoglobin and other negatively charged proteins and then were concentrated to 175 $\mu$L. Colchicine-binding assays of this concentrated material (see Experimental Procedures) showed that it contained $(3.25 \pm 0.3) \times 10^{-7}$ M tubulin dimer, under the assumption that the tubulin of RRL binds colchicine with the same affinity and speed as does bovine tubulin. This concentration corresponds to $(5.7 \pm 1) \times 10^{-9}$ M tubulin in RRL, or $5.7 \pm 1$ $\mu$g in the total 10 mL folding-mixture. A second approach was to carry out an immunoblot of SDS-gel lanes containing the concentrated total protein from a known volume of RRL together with lanes containing known amounts of bovine tubulin (Figure 6). Amounts of total rabbit tubulin were obtained by comparison of the integrated intensities of bands produced by the RRL to those obtained from the bovine standards on the same blot. Because the method presumes that roughly equal intensities are obtained from equal amounts of protein, the blot was probed with a mixture of two broad-spectrum antibodies in an attempt to minimize possible isotype-related differences in reactivity (see Experimental Procedures). The amount measured ($0.47 \pm 0.1$ $\mu$g/mL of RRL) corresponds to $4.7 \pm 1$ $\mu$g of total tubulin in 10 mL of RRL, in good agreement with the estimate obtained from colchicine binding. From these two estimates, an absolute upper limit can be calculated for the fraction of rabbit tubulin that could possibly be present in the microtubules: even if all the tubulin in the entire 10 mL of RRL present in the folding-mixture were incorporated into the microtubules, it could compose no more than about 20% of the tubulin in the 27 $\mu$g of microtubules finally obtained.

## DISCUSSION

The results demonstrate that recombinantly expressed mammalian tubulin, composed largely of a single α-chain and a single $\beta$-chain, when folded in vitro and subjected to assembly-promoting conditions, will form microtubules of apparently normal structure. Analysis by isoelectric focusing of the two tubulin chains which compose these microtubules yielded no evidence of modifications that alter their isoelectric pH. These two findings, taken together, show that the mixture of isoforms which is present in mammalian cells is not necessary for the formation of microtubules. This result implies that the functional reasons for their presence are likely to be found elsewhere, for instance in modulation of the affinity of microtubules for proteins and small ligands. Because of the great difficulties posed by biochemical separations of native tubulins, functional study (*17*−*21, 23, 24, 27, 29, 30*) has thus far been restricted to dimers composed, for instance, of a single $\beta$-chain sequence paired with the full mixture of α-chain sequences, with the additional complexity arising from the posttranslational modifications characteristic of each sequence. By bypassing those limitations, this study demonstrates the feasibility of an alternative approach.

Control experiments to measure the possible degree of contamination of the recombinant tubulin by tubulin from RRL were rendered difficult by the minuscule concentration and unknown isoform-composition of the tubulin in RRL.[2] The results which do not rely heavily on the specificity or quantitation of antibody reactions are most secure. It is clear from the silver-stained gel in Figure 5B that the great majority of the tubulin in the microtubules must be recombinant protein, correctly folded. Furthermore, to produce the single-component IEF patterns of Figure 5C,D, a hypothetical contaminant would need to have the same isoelectric pH as the recombinant protein, and for both α- and $\beta$-chains, an

[2] The isotypes and isoforms of tubulin present in RRL are not known. Early work established that the M$\beta$1 isotype and the c$\beta$1 isotypes [both Class VI (*1*)] were found primarily in mouse spleen and chicken erythrocytes and bone marrow, respectively (*2, 3*). But this "hematopoietic" tubulin, although largely confined to spleen and marrow, is not the only one present; sequences M$\beta$2, M$\beta$3, and M$\beta$5 are also found there in equal or greater amounts (*2, 4*). Recent work, in fact, shows that expression of M$\beta$1 is apparently confined to the platelet-producing cell lineage in mouse, appearing in megakaryocytes, proplatelets, and platelets (*5*). The reticulocytes, of the erythrocyte-producing lineage, must contain other isotypes. The mixture of cells in the reticulocyte-rich blood from which RRL is prepared must include reticulocytes, platelets, and erythrocytes, among others. It is reasonable, therefore, to expect a mixture of isotypes.

unlikely possibility. The results, however, do not rule out the possibilities that some contaminating tubulin from RRL may be present in the microtubules or that some RRL tubulin, perhaps by acting as a nucleating center, may be important for microtubule assembly in this system.

Several results obtained early in the investigation were exploited to increase the total yield of the folding reaction. The maximal concentration of urea tolerated by the folding system was found to increase from approximately 0.15 M to approximately 0.4 M when trimethylamine oxide was present, allowing addition of increased amounts of substrate. The near-proportionality between the overall yield of the folding reaction and the concentration of unfolded tubulin added to the folding-mixture (Figure 2) must be the net result of several processes, some of them competing. For instance, increasing the concentration of unfolded protein added to the folding-mixture may cause it to aggregate more rapidly once it is diluted, removing it from accessibility to the chaperone system [a situation sometimes termed "kinetic partitioning", (*82*)]. But the higher concentration may also lead to an increase in the rate of a kinetically limiting step in the folding reaction, or to displacement of the reaction's equilibrium position toward larger ratios of folded to unfolded protein, or to both effects. The tubulin concentration employed (3 mg/mL) was the maximum attainable with the methods at hand; no full-scale optimization of the added amount has yet been attempted. Centrifugal treatment of the RRL before the start of the folding reaction, and chromatographic isolation of tubulin before its final purification by polymerization, also improved yield, perhaps by removing unknown inhibitory substances. Finally, in agreement with prior qualitative findings (*55, 83*), addition of GMPCPP reduced the apparent critical concentration to near 50 $\mu$g/mL, as determined by centrifugal assay, and facilitated efficient final purification of tubulin by polymerization. Unlike paclitaxel, another assembly promoter which might have been used, this nucleotide analogue can easily be displaced by the natural ligand GTP to allow subsequent functional study (*55*). When 30 mg of tubulin chains (8 mg of $\alpha$-chain and 22 mg of $\beta$-chain) was added to a 10 mL folding reaction, 27 $\mu$g was recovered as microtubules. For comparison, the amount of folded tubulin obtained from in vitro translation of mRNA followed by folding in RRL by Yaffe et al. (*31*) corresponded to a maximum of 2−4 $\mu$g in a 10 mL folding-mixture.[3] Thus, when account is taken of the 200-fold increase in scale, the current folding conditions are seen to give a minimum 7−14-fold increase in efficiency over those previously employed.[4] Further refinement of the system to increase both efficiency and mass yield appears distinctly possible.

In the current experiments, only a single $\alpha$-chain sequence and a single $\beta$-chain sequence were investigated, but there appears to be no reason that other tubulins cannot be explored. When combined with knowledge of the high-resolution structure of tubulin (*7*), these results open the way to in vitro structure−function studies (e.g., drug binding, MAP binding, recognition of molecular motors, etc.) of tubulins that have been altered by site-directed mutagenesis. Preparing native tubulin in this way has an advantage over isolation of genetically modified tubulins from microorganisms (*84, 85*) because it allows in vitro study even of mutant polypeptides which may be lethal if present in a host organism. Yet another potential advantage of recombinant tubulins folded in vitro is the possibility that fully homogeneous tubulin could provide well-ordered crystals for structural studies.

[3] Yaffe et al. (*6*) obtained 0.9−1.8 pmol of full-length polypeptide in a 50 $\mu$L reaction mixture, of which approximately 50% was free $\beta$-chain, uncomplexed with components of the folding machinery. Of that amount, approximately 22% could be recovered after chromatographic purification, as indicated in Table 1 of the report. If all this material entered into dimers, and if all the dimers could polymerize, this would amount to 0.1−0.2 pmol, or 0.01−0.02 $\mu$g. When scaled to 10 mL for comparison with the current results, this would be 2−4 $\mu$g of tubulin.

[4] Because unfolded recombinant tubulin is so easily and inexpensively produced, efficiency in its use (in the sense of folding a large *fraction* of the material added to the folding-mixture) was not a primary concern in this work. Greater fractional yields of folded protein could be achieved, at the cost of mass yield, by reducing the mass of recombinant tubulin added to the folding-mixture.

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## REFERENCES

1. Sullivan, K. F. (1988) *Annu. Rev. Cell Biol 4*, 687−716.
2. Wang, D., Villasante, A., Lewis, S. A., and Cowan, N. J. (1986) *J. Cell Biol. 103*, 1903−1910.
3. Murphy, D. B., Wallis, K. T., Machlin, P. S., Ratrie, H. d., and Cleveland, D. W. (1987) *J. Biol. Chem. 262*, 14305−14312.
4. Lewis, S. A., Lee, M. G., and Cowan, N. J. (1985) *J. Cell Biol. 101*, 852−861.
5. Lecine, P., Italiano, J. E., Jr., Kim, S. W., Villeval, J. L., and Shivdasani, R. A. (2000) *Blood 96*, 1366−1373.
6. Yaffe, M. M., Farr, G. W., and Sternlicht, H. (1988) *J. Biol. Chem. 263*, 16023−16031.
7. Nogales, E., Wolf, S. G., and Downing, K. H. (1998) *Nature 391*, 199−203.
8. Nogales, E., Whittaker, M., Milligan, R. A., and Downing, K. H. (1999) *Cell 96*, 79−88.
9. Stewart, Z. A., Mays, D., and Pietenpol, J. A. (1999) *Cancer Res. 59*, 3831−3837.
10. Wang, L. G., Liu, X. M., Kreis, W., and Budman, D. R. (1999) *Cancer Chemother. Pharmacol. 44*, 355−361.
11. Jordan, M. A., and Wilson, L. (1998) *Curr. Opin. Cell Biol. 10*, 123−130.
12. Field, D. J., and Lee, J. C. (1985) *Anal. Biochem. 144*, 584−592.
13. Lee, J. C., Field, D. J., George, H. J., and Head, J. (1986) *Ann. N.Y. Acad. Sci. 466*, 111−128.
14. Williams, R. C., Jr., Shah, C., and Sackett, D. (1999) *Anal. Biochem 275*, 265−267.
15. MacRae, T. H. (1997) *Eur. J. Biochem. 244*, 265−278.
16. Laferriere, N. B., MacRae, T. H., and Brown, D. L. (1997) *Biochem. Cell Biol. 75*, 103−117.
17. Banerjee, A., Roach, M. C., Treka, P., and Luduena, R. F. (1990) *J. Biol. Chem. 265*, 1794−1799.
18. Banerjee, A., and Luduena, R. (1992) *J. Biol. Chem. 267*, 13335−13339.
19. Panda, D., Miller, H. P., Banerjee, A., Luduena, R. F., and Wilson, L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11358−11362.

20. Banerjee, A., Engelborghs, Y., D'Hoore, A., and Fitzgerald, T. J. (1997) *Eur. J. Biochem. 246*, 420−424.
21. Hutchens, J. A., Hoyle, H. D., Turner, F. R., and Raff, E. C. (1997) *Mol. Biol. Cell 8*, 481−500.
22. Wilson, P. G., and Borisy, G. G. (1997) *Bioessays 19*, 451−454.
23. Roskams, A. J., Cai, X., and Ronnett, G. V. (1998) *Neuroscience 83*, 191−200.
24. Banerjee, A., and Kasmala, L. T. (1998) *Biochem. Biophys. Res. Commun. 245*, 349−351.
25. Redeker, V., Rossier, J., and Frankfurter, A. (1998) *Biochemistry 37*, 14838−14844.
26. Kavallaris, M., Burkhart, C. A., and Horwitz, S. B. (1999) *Br. J. Cancer 80*, 1020−1025.
27. Saragoni, L., Hernandez, P., and Maccioni, R. B. (2000) *Neurochem. Res. 25*, 59−70.
28. Luduena, R. F. (1998) *Int. Rev. Cytol. 178*, 207−275.
29. Banerjee, A., Roach, M. C., Trcka, P., and Luduena, R. F. (1992) *J. Biol. Chem. 267*, 5625−5630.
30. Lobert, S., Frankfurter, A., and Correia, J. J. (1995) *Biochemistry 34*, 8050−8060.
31. Yaffe, M. B., Levinson, B. S., Szasz, J., and Sternlicht, H. (1988) *Biochemistry 27*, 1869−1880.
32. Yaffe, M. B., Farr, G. W., and Sternlicht, H. (1989) *J. Biol. Chem. 264*, 19045−19051.
33. Farr, G. W., Yaffe, M. B., and Sternlicht, H. (1989) *J. Biol. Chem. 264*, 19045−19051.
34. Yaffe, M. B., Farr, G. W., Miklos, D., Horwich, A. L., Sternlicht, M. L., and Sternlicht, H. (1992) *Nature (London) 358*, 245−248.
35. Kubota, H., Hynes, G., and Willison, K. (1995) *Eur. J. Biochem. 230*, 3−16.
36. Melki, R., Rommelaere, H., Leguy, R., Vandekerckhove, J., and Ampe, C. (1996) *Biochemistry 35*, 10422−10435.
37. Melki, R., Batelier, G., Soulie, S., and Williams, R. C., Jr. (1997) *Biochemistry 36*, 5817−5826.
38. Llorca, O., Smyth, M. G., Carrascosa, J. L., Willison, K. R., Radermacher, M., Steinbacher, S., and Valpuesta, J. M. (1999) *Nat. Struct. Biol. 6*, 639−642.
39. Melki, R., and Cowan, N. J. (1994) *Mol. Cell. Biol. 14*, 2895−2904.
40. Archer, J. E., Vega, L. R., and Solomon, F. (1995) *Cell 82*, 425−434.
41. Farr, G. W., Scharl, E. C., Schumacher, R. J., Sondek, S., and Horwich, A. L. (1997) *Cell 89*, 927−937.
42. Archer, J. E., Magendantz, M., Vega, L. R., and Solomon, F. (1998) *Mol. Cell. Biol. 18*, 1757−1762.
43. Yokota, S., Yanagi, H., Yura, T., and Kubota, H. (1999) *J. Biol. Chem. 274*, 37070−37078.
44. Thulasiraman, V., Yang, C. F., and Frydman, J. (1999) *EMBO J. 18*, 85−95.
45. Hansen, W. J., Cowan, N. J., and Welch, W. J. (1999) *J. Cell Biol. 145*, 265−277.
46. Lewis, S., Tian, G., and Cowan, N. (1997) *Trends Cell Biol. 7*, 479−484.
47. Liang, P., and MacRae, T. H. (1997) *J. Cell Sci. 110*, 1431−1440.
48. Cowan, N. J., and Lewis, S. A. (1999) *Nat. Struct. Biol. 6*, 990−991.
49. Gao, Y., Vainberg, I. E., Chow, R. L., and Cowan, N. J. (1993) *Mol. Cell. Biol. 13*, 2478−2485.
50. Gao, Y., Melki, R., Walden, P. D., Lewis, S. A., Ampe, C., Rommelaere, H., Vandekerckhove, J., and Cowan, N. J. (1994) *J. Cell Biol. 125*, 989−996.
51. Zabala, J. C., and Cowan, N. J. (1992) *Cell Motil. Cytoskeleton 23*, 222−230.
52. Fontalba, A., Paciucci, R., Avila, J., and Zabala, J. C. (1993) *J. Cell Sci. 106*, 627−632.
53. Williams, R. C., Jr., and Lee, J. C. (1982) *Methods Enzymol. 85*, 376−385.
54. Hyman, A. A., Salser, S., Drechsel, D. N., Unwin, N., and Mitchison, T. J. (1992) *Mol. Biol. Cell 3*, 1155−1167.
55. Vulevic, B., and Correia, J. J. (1997) *Biophys. J. 72*, 1357−1375.
56. Hagel, P., Gerding, J. J., Fieggen, W., and Bloemendal, H. (1971) *Biochim. Biophys. Acta 243*, 366−373.
57. Villasante, A., Wang, D., Dobner, P., Dolph, P., Lewis, S. A., and Cowan, N. J. (1986) *Mol. Cell. Biol. 6*, 2409−2419.
58. Jackson, R. J., and Hunt, T. (1983) *Methods Enzymol. 96, Part J*, 50−74.
59. Sackett, D. L. (1997) *Am. J. Physiol. 273*, R669−676.
60. Baskakov, I., Wang, A., and Bolen, D. W. (1998) *Biophys. J. 74*, 2666−2673.
61. Gao, Y., Thomas, J. O., Chow, R. L., Lee, G.-H., and Cowan, N. J. (1992) *Cell 69*, 1043−1050.
62. Cowan, N. J. (1998) *Methods Enzymol. 290*, 230−241.
63. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
64. Zor, T., and Selinger, Z. (1996) *Anal. Biochem. 236*, 302−308.
65. Rabilloud, T., Adessi, C., Giraudel, A., and Lunardi, J. (1997) *Electrophoresis 18*, 307−316.
66. Andreu, J. M., and Timasheff, S. N. (1982) *Biochemistry 21*, 6465−6476.
67. Skoufias, D. A., Wilson, L., and Detrich, H. W., III (1992) *Cell Motil. Cytoskeleton 21*, 272−280.
68. Lobert, S., and Correia, J. J. (1994) *Electrophoresis 15*, 930−931.
69. Viklicky, V., Draber, P., Hasek, J., and Bartek, J. (1982) *Cell Biol. Int. Rep. 6*, 725−731.
70. Draber, P., Lagunowich, L. A., Draberova, E., Viklicky, V., and Damjanov, I. (1988) *Histochemistry 89*, 485−492.
71. Linhartova, I., Draber, P., Draberova, E., and Viklicky, V. (1992) *Biochem. J. 288*, 919−924.
72. Linhartova, I., Draberova, E., Viklicky, V., and Draber, P. (1993) *FEBS Lett. 320*, 79−82.
73. Smertenko, A., Blume, Y., Viklicky, V., Opatrny, Z., and Draber, P. (1997) *Planta 201*, 349−358.
74. Binder, L. I., Frankfurter, A., and Rebhun, L. I. (1986) *Ann. N.Y. Acad. Sci. 466*, 145−166.
75. Caccamo, D. V., Katsetos, C. D., Frankfurter, A., Collins, V. P., Vandenburg, S. R., and Herman, M. M. (1989) *Neuropathol. Appl. Neurobiol. 15*, 389−405.
76. Caccamo, D., Katsetos, C. D., Herman, M. M., Frankfurter, A., Collins, V. P., and Rubinstein, L. J. (1989) *Lab. Invest. 60*, 390−398.
77. Lee, M. K., Tuttle, J. B., Rebhun, L. I., Cleveland, D. W., and Frankfurter, A. (1990) *Cell Motil. Cytoskeleton 17*, 118−132.
78. Gildersleeve, R. F., Cross, A. R., Cullen, R. E., Fagen, A. P., and Williams, R. C., Jr. (1992) *J. Biol. Chem. 267*, 7995−8006.
79. Field, D. J., Collins, R. A., and Lee, J. C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4041−4045.
80. Grasso, J. (1966) *Anat. Rec. 156*, 397−413.
81. Koury, S. T., Koury, M. J., and Bondurant, M. C. (1989) *J. Cell Biol. 109*, 3005−3013.
82. Weissman, J. S., Kashi, Y., Fenton, W. A., and Horwich, A. L. (1994) *Cell 78*, 693−702.
83. Hyman, A. A., Salser, S., Drechsel, D. N., Unwin, N., and Mitchison, T. J. (1992) *Mol. Biol. Cell 3*, 1155−1167.
84. Sage, C. R., Davis, A. S., Dougherty, C. A., Sullivan, K., and Farrell, K. W. (1995) *Cell Motil. Cytoskeleton 30*, 285−300.
85. Davis, A., Sage, C. R., Wilson, L., and Farrell, K. W. (1993) *Biochemistry 32*, 8823−8835.
86. Tian, G., Lewis, S. A., Feierbach, B., Stearns, T., Rommelaere, H., Ampe, C., and Cowan, N. J. (1997) *J. Cell Biol. 138*, 821−832.
87. Tian, G., Huang, Y., Rommelaere, H., Vandekerckhove, J., Ampe, C., and Cowan, N. J. (1996) *Cell 86*, 287−296.
88. Murphy, D. B., Grasser, W. A., and Wallis, K. T. (1986) *J. Cell Biol. 102*, 628−635.


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