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Isolation and Partial Characterization of α- and β-Tubulin from Outer Doublets of Sea-Urchin Sperm and Microtubules of Chick-Embryo Brain

(*Strongylocentrotus*/gel electrophoresis/protein sequencing/evolution)

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ABSTRACT **Two kinds of tubulin (α and β) have been described in microtubules from many different systems. In this study a discontinuous acrylamide-gel system containing sodium dodecyl sulfate was used to separate milligram quantities of α- and β-tubulin from microtubules of chick-embryo brain and from outer doublets of sea-urchin sperm. The isolated tubulins were characterized by peptide mapping and automated sequencing of the first 25 NH_2-terminal amino acids. Our results show that α- and β-tubulin are related but distinctly different proteins and that each one has been highly conserved in the course of evolution.**

The structural subunit of microtubules is a 100,000–120,000 molecular weight dimer of two 55,000–60,000 molecular weight monomers (1). Recent evidence suggests that there may be at least two kinds of tubulin monomers in microtubules, although it is not clear how they are arranged within the microtubular structure. When microtubules from various sources are solubilized and subjected to electrophoresis on acrylamide gels containing urea or Na dodecyl sulfate, two bands are obtained which contain proteins differing in amino-acid composition and in cyanogen bromide and tryptic peptide maps (2–6).

Despite the morphological similarity of microtubules from different sources, it is not known how closely tubulin monomers from different organisms and different types of microtubules resemble each other. Studies using colchicine have shown that microtubules from many different sources bind to this drug with similar affinities, implying the conservation of at least one common site (4, 7–9). Immunological studies have given ambiguous results; some of these studies suggest the presence of a microtubular antigen in a wide variety of organisms (10, 11) and another study suggests that microtubular antigens may vary widely from one organism to another (12).

A major obstacle to analyzing the differences between the two forms of tubulin and between tubulins from different sources has hitherto been the difficulty in separating and isolating them in milligram quantities. We report here a method for isolating preparative amounts of each tubulin species on a discontinuous Na dodecyl sulfate gel system, using as sources microtubules from embryonic chick brain and outer doublets of sea-urchin sperm tails. The amino-acid sequences of the two tubulins differ considerably, but relatedness is apparent from their primary structure. Comparison of corresponding tubulin species from each organism indicated that the primary structure of each type of tubulin is strongly conserved in evolution.

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MATERIAL AND METHODS

Preparation of Microtubules. Outer doublets were isolated from sperm flagella of several hundred male sea urchins of the species *Strongylocentrotus purpuratus* by the method of Stephens (13). Brain microtubule protein was the generous gift of Dr. Leslie Wilson. It was prepared from 16- to 18-day-old chick-embryo brains by the method of Bryan and Wilson (4).

Protein Was Determined by the method of Lowry *et al.* (14) as modified by Bailey (15), with bovine-serum albumin as a standard. The intensity of the color that developed was measured on a Klett–Summerson photoelectric colorimeter with a red filter.

Carboxymethylation. Protein samples were carboxymethylated by reaction with sodium iodoacetate (16); they were then dialyzed exhaustively in the dark against distilled water and lyophilized.

Polyacrylamide Gel Electrophoresis. Continuous urea system. Samples were dissolved in 8 M urea–30 mM Tris·HCl (pH 7.8) and run at 200 V for 2 hr on 4.5% polyacrylamide gels, which were 8 M in urea (3). The gels had been previously subjected to electrophoresis at 200 V for 45 min.

Discontinuous urea system. This system was based on that of Davis (17) as modified by Yang and Criddle (18) and Shuster (19). The samples were incubated at 100° for 2–3 min in 1% Na dodecyl sulfate–0.01 M Tris·HCl (pH 8.0) and then dialyzed for 6–20 hr at 24° against 10% sucrose, containing 59 mM Tris-phosphate (pH 7.2) before they were layered on the gel. The running buffer was routinely 52 mM Tris–53mM glycine–0.03% Na dodecyl sulfate. The acrylamide to bis ratio in the lower gel was 32:1, and in the upper 4:1.

Continuous Na dodecyl sulfate–urea system. The system of Swank and Munkres (20) was used for mapping the products of cyanogen bromide cleavage. Gels from all these systems were stained for 6–12 hr in a solution of 0.03% Coomassie blue in methanol–acetic acid–water (45:9:46) and then rinsed in methanol–acetic acid–water (5:7.5:87.5).

Preparative Polyacrylamide-Gel Electrophoresis. The discontinuous Na dodecyl sulfate system was adapted for use with a Buchler model 200 polyprep by adopting suggestions of Chrambach (21) and Rodbard and Chrambach (22). The

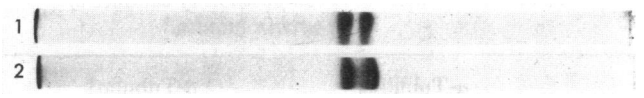

FIG. 1. Electrophoresis of microtubule proteins on continuous urea gels. Samples were run on 4.8% gels as follows: (*1*) Carboxymethylated microtubule protein of chick-embryo; (*2*) carboxymethylated outer doublet protein of sea-urchin sperm. The gels were stained with Coomassie blue. (Origins are at the *left*.)

lower gel was poured 2 days before electrophoresis and layered with distilled water. After the gel hardened, the distilled water was replaced by lower gel buffer. The lower electrophoresis buffer was 0.1 M Tris·HCl (pH 8.1). The upper electrophoresis buffer was 52 mM Tris–35 mM glycine–0.03% Na dodecyl sulfate. The first elution buffer, used until the tracking dye came off the gel, was 0.375 M Tris·HCl (pH 8.9). The second elution buffer, used thereafter, was 1.45 M Tris·HCl (pH 9.45) made 0.73 M in sucrose. The elution rate was 1–2 ml/min. Phenol red was the tracking dye. The current was 40 mA. Fractions were collected and their absorbance at 280 nm measured; fractions were pooled, concentrated with a Diaflo PM 10 membrane filter, dialyzed exhaustively against distilled water, and lyophilized.

Amino-Acid Analysis. Lyophilized samples of protein were hydrolyzed in 6 N HCl at 110° for 22–24 hr under reduced pressure. They were then dried in a desiccator and analyzed on a Beckman model 120 amino-acid analyzer by standard procedures (23, 24).

Cyanogen Bromide Cleavage. Carboxymethylated samples were reacted for 24 hr at room temperature with 0.945 M cyanogen bromide in 70% formic acid (25). The molar ratio of cyanogen bromide to methionine residues in the sample was in excess of 100:1. The reaction mixtures were diluted with distilled water and lyophilized.

NH_2-Terminal Analysis. Samples were reacted with dansyl chloride and hydrolyzed according to Gros and Labouesse (26). Certain samples were subjected to the microanalytic dansyl-Edman procedure of Weiner *et al.* (27).

Sequence Analysis. Approximately 2–8 mg of protein were placed in the reaction cup of a Beckman model 890 automated sequencer and dissolved in heptafluorobutyric acid. The sample was then subjected to automated Edman degradation (28) using the fast quadrol program (Beckman sequencer manual). The consecutive amino acids were collected as their thiazolinone derivatives, converted to their phenylthiohydantoin derivatives, and identified by gas chromatography

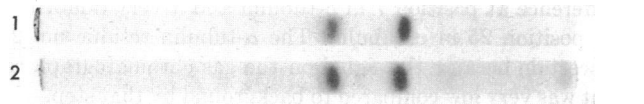

FIG. 2. Electrophoresis of microtubule proteins on discontinuous Na dodecyl sulfate gels. 7% gels, 9 cm long, were run in the discontinuous Na dodecyl sulfate system until the bromphenol blue tracking dye was 1 cm from the bottom of the gel: (*1*) Carboxymethylated microtubule protein of chick-embryo brain; (*2*) carboxymethylated outer doublet protein of sea-urchin sperm. The gels were stained with Coomassie blue.

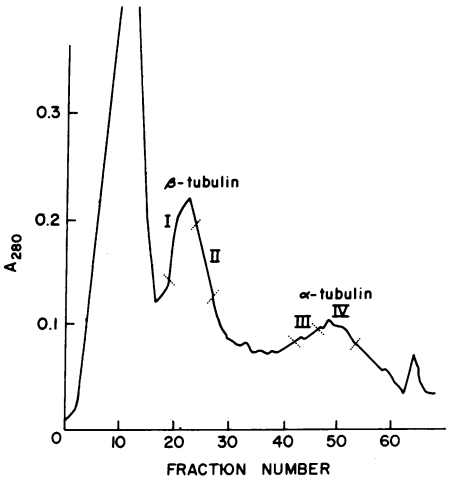

FIG. 3. Separation on the polyprep of α- and β-tubulin from outer doublets of sea-urchin sperm. 39.3 mg of carboxymethylated microtubule protein of outer doublets of *S. purpuratus* sperm were run on a Buchler polyprep 200 using the discontinuous Na dodecyl sulfate gel system. The eluted fractions were collected and their absorptions at 280 nm were determined. *Roman numerals* are used to label the fractions that were pooled for subsequent analytical electrophoresis (Fig. 4).

over a column packed with Chromasorb-W coated with SP 400. Aliquots of each phenylthiohydantoin derivative were reacted with *N,O*-bis(trimethylsilyl)acetamide before chromatography. Certain residues were hydrolyzed under reduced pressure in 6 N HCl containing 1:2000 2-mercaptoethanol for 24 hr at 130°. The hydrolyzed residues were identified on the amino-acid analyzer.

RESULTS

Separation of alpha- and beta-tubulin

Continuous Urea Gel System. Carboxymethylated sperm outer doublets and brain microtubules each migrated as two bands. In accordance with the nomenclature of Bryan and Wilson (4), who also used this gel system, we shall hereafter refer to the faster-moving of the two bands as β-tubulin and to the slower-moving one as α-tubulin (Fig. 1).

Discontinuous Na Dodecyl Sulfate Gel System. In this system, proteins tended to separate according to molecular weight, although quantitative deviations occurred among the faster-

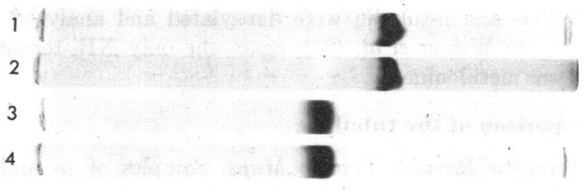

FIG. 4. Electrophoretic analysis of α- and β-tubulin samples of sea-urchin outer doublets isolated by preparative electrophoresis. Pooled fractions (Fig. 3) were run on 7% discontinuous Na dodecyl sulfate gels.

Gel no.	*Fraction no.*	*Type of tubulin in the fraction*
1	I	β-Tubulin
2	II	β-Tubulin
3	III	α-Tubulin
4	IV	α-Tubulin

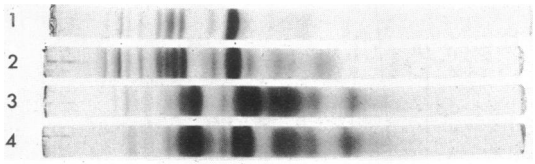

FIG. 5. Cyanogen bromide peptide maps of carboxymethylated tubulins. Samples of α- and β-tubulin were digested with cyanogen bromide, and run on the continuous Na dodecyl sulfate–urea system, as follows: (*1*) Digest of α-tubulin of chick-embryo brain; (*2*) digest of α-tubulin of sea-urchin sperm; (*3*) digest of β-tubulin of chick-embryo brain; (*4*) digest of β-tubulin of sea-urchin sperm.

moving proteins. Carboxymethylated microtubules migrated as two well-separated bands, with mobilities relative to the tracking dye of 0.55 and 0.68 (Fig. 2).

The two gel systems were correlated by running samples on the continuous urea gel system; these gels were then sliced and the slices were analyzed on the discontinuous Na dodecyl sulfate gel system. These experiments were done separately for the outer doublet of sea-urchin sperm and for the chick-brain proteins. In each case, the results showed that β-tubulin was the faster-moving species in both gel systems, and α-tubulin was the slower-moving one.

Preparative Acrylamide-Gel Electrophoresis. Samples of carboxymethylated microtubules were subjected to electrophoresis on the polyprep as shown in Figs. 3 and 4. In a typical run, 5 mg of each tubulin could be obtained in relatively pure form from 30 mg of carboxymethylated microtubules. α- and β-tubulin were thus isolated from both microtubules of chick-embryo brain and outer doublet microtubules of sea-urchin sperm.

Amino-acid analysis

The amino-acid compositions of α- and β-tubulin from *S. purpuratus* outer doublets were determined and are given in Table 1. The amino-acid compositions are generally similar to those of α- and β-tubulin from chick-embryo brain (4).

NH_2-terminal analysis

Carboxymethylated microtubule protein from both sea-urchin outer doublets and chick-embryo brain was dansylated (26), hydrolyzed, and chromatographed either on silica gel-coated glass plates or on polyamide-coated plates. In each case the only NH_2-terminus seen was methionine. When purified sea-urchin α- and β-tubulin were dansylated and analyzed according to Weiner *et al.* (27), again the only NH_2-terminus seen was methionine.

Comparison of the tubulins

Cyanogen Bromide Peptide Maps. Samples of α- and β-tubulin, from both outer doublets of sea-urchin sperm and microtubules of chick-embryo brain, which had been separated on the polyprep, were digested with cyanogen bromide and analyzed on the continuous Na dodecyl sulfate–urea system of Swank and Munkres (20). The resulting peptide maps showed that the two α-tubulins were very similar to each other and that the two β-tubulins were also quite similar to each other (Fig. 5). In the case of α-tubulin, nine bands were common to both chick and sea-urchin maps, while five were unique to the former and nine to the latter. In the case of β-tubulin, nine bands were common to both; six were unique to chick brain and seven to sea urchin. A band was counted as common to two maps if it was present in both maps at the same position and was equally sharply defined in both maps. The differences described were reproducible. Thus, this experiment implies that corresponding tubulins in the two organisms are more closely related to each other than either α-tubulin is related to either β-tubulin.

TABLE 1. *Amino-acid composition of samples of sea-urchin tubulin**

amino acid	α-Tubulin† (g amino acid/100 g protein)	α-Tubulin† (moles amino acid/55,000 molecular weight)	β-Tubulin† (g amino acid/100 g protein)	β-Tubulin† (moles amino acid/55,000 molecular weight)
Lys	5.66	24.3	4.93	21.1
His	3.53	14.2	2.82	11.3
Arg	7.41	26.1	6.65	23.4
Cys‡	2.88	9.8	2.01	6.9
Asp	10.13	48.4	11.41	54.5
Thr	5.41	29.4	5.79	31.5
Ser	3.31	20.9	3.83	24.2
Glu	16.21	69.0	16.25	69.2
Pro	4.48	25.3	4.89	27.7
Gly	4.22	40.7	4.40	42.4
Ala	5.23	40.5	4.44	34.4
Val	5.96	33.1	5.96	33.1
Met	2.34	9.8	3.96	16.6
Ile	5.07	24.6	3.90	18.9
Leu	7.30	35.5	7.48	36.4
Tyr	5.23	17.6	4.98	16.8
Phe	5.64	21.1	6.31	23.6
Trp	ND	ND	ND	ND

* Lyophilized samples were hydrolyzed in sealed tubes in the presence of 6 N HCl at 110° for 23 hr.
† Figures represent the average of two analyses.
‡ Cysteine determined as carboxymethylcysteine.
ND, not determined

Sequence Analysis. The NH_2-terminal sequences of the four tubulins are shown in Fig. 6. The two sea-urchin tubulins were sequenced twice through position 9 and once through 25. The two chick-brain tubulins were sequenced twice through position 20 and once through 30. There are thus, several uncertain identifications due to insufficient repeats over the same sequence. Nevertheless, the sequences reveal a very close resemblance between corresponding tubulins from chick-embryo brain and sea-urchin sperm. The only differences observed between the tubulins of the two organisms are a definite difference at position 7 in β-tubulin and a very doubtful one at position 25 in α-tubulin. The α-tubulin residue no. 25 is uncertain because the signal on the gas chromatograph readout was very low compared to background by that step.

DISCUSSION

The information we have obtained on the sequences of α- and β-tubulin confirms what the work of Bryan and Wilson (4), Fine (5), and Feit *et al.* (2) strongly implied; namely, that α- and β-tubulins are different proteins with different amino-acid sequences. Sequence studies and cyanogen bromide pep-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Chick-brain α-tubulin:	Met-	Arg-	Glx-	Ser?	Ile-	Ser?	Ile-	His-	Val-	Thr-	Gln-	Ala-	Thr-	Val-	Gln-
Sea-urchin α-tubulin:	Met-	Arg-	Glu-	Ser?	Ile-	Ser?	Ile-	His-	Val-	Thr-	Glx-	Ala-	Thr-	Val-	Glx-
Chick-brain β-tubulin:	Met-	Arg-	Glu-	Ile-	Val-	His-	Ile-	Gln-	Ala-	Thr-	Gln-	Ser-	Thr-	Asx-	Gln-
Sea-urchin β-tubulin:	Met-	Arg-	Glu-	Ile-	Val-	His-	Met-	Glx-	Ala-	Thr-	Glx-	Ser-	Thr-	Asx-	Glx-

	16	17	18	19	20	21	22	23	24	25
Chick-brain α-tubulin:	Ile-	Thr-	Asx-	Ala-	Ser?	?	-Glx-	Leu-	Tyr-	Ser?
Sea-urchin α-tubulin:	Ile-	Thr-	Asx-	Ala-	Ser?	?	-Glx-	Leu-	Tyr-	Ala?
Chick-brain β-tubulin:	Ile-	Thr-	Ala-	?	-Phe-	Trp?	Glx-	Val-	Ile-	Ser?
Sea-urchin β-tubulin:	Ile-	Thr-	Ala-	?	-Phe-	?	?-	Val-	Ile-	Ser?

FIG. 6. Amino-acid sequences of NH_2-terminal regions of α- and β-tubulin from chick brain and from the outer doublet microtubules of sea-urchin sperm.

tide maps indicate that the differences between the proteins are considerable.

Evidence presented here shows that the α-tubulins from chick-embryo brain and sea-urchin sperm closely resemble each other, that the β-tubulins from these two sources do likewise, and that these similarities are much greater than those between α- and β-tubulin within either species.

The similarity between corresponding tubulins is demonstrated most dramatically in the NH_2-terminal region from sequence data. No differences between α-tubulin of chick-embryo brain and sea-urchin sperm are apparent in the first 24 residues. The observed difference at the 25th residue is very questionable. In the case of β-tubulin, the chick-brain protein has an isoleucine residue at position 7 where the sea urchin has methionine, which is the only difference observed in the first 25 residues. This similarity in the NH_2-terminal region and in the cyanogen bromide peptide maps suggests that the two α-tubulins and likewise the two β-tubulins are very much alike along the entire polypeptide chain.

Based on the sequences shown in Fig. 6, α- and β-tubulin are related proteins. They appear to be identical at 11 of the first 25 positions; i.e., at positions 1, 2, 3, 10, 11, 13, 15, 16, 17, 22, and possibly 25. Furthermore, out of the 14 positions where they differ, 9 of these can be accounted for by single base-pair changes (at positions 4, 5, 6, 7, 8, 9, 12, 20, and 23). The changes at positions 14 and 18 would require one or two mutations, depending on whether the aspartic acid is amidated. Only at position 24, where one is isoleucine and the other tyrosine, is a minimum of two base-pair changes definitely necessary. These similarities are more than would be expected by chance and, therefore, α- and β-tubulin very likely evolved from a single protein, originally possibly serving a structural function. The gene coding for this protein eventually could have duplicated, allowing the two tubulins to diverge fairly rapidly until sequences similar to α- and β-tubulin arose. Microtubules formed from these two proteins must have been similar to the microtubules of present-day organisms, and thus, having reached this point, the rate of change in tubulins must have slowed considerably.

Out of 32 unambiguously identified positions in the amino-acid sequences of the two tubulins† there is only one position—number 7 in β-tubulin—where the corresponding tubulins from chick brain and sea-urchin sperm differ. Assuming that the rest of the amino-acid sequences are as similar as are the NH_2-terminal regions, we can estimate that the tubulins have changed at 1 out of every 32 positions since the time of the common ancestor of chordates and echinoderms. The existence of a possible echinoderm in a pre-Cambrian fossil fauna (29) and the possible presence of four echinoderm subphyla in the Cambrian period (30) imply a fairly long pre-Cambrian evolutionary history for the echinoderms. Seven hundred million years is thus a conservative estimate for the time that has elapsed since the echinoderm and chordate lines diverged. These assumptions yield an evolutionary rate of 0.45 acceptable point mutations per hundred residues per hundred million years, which suggests that the tubulins are more conservative than any other protein family so far studied except the histones (31).

It is generally assumed that evolutionary conservatism of a protein reflects the number and degree of constraints involved in the functioning of that protein. This being the case, the tubulins must be subject to severe constraints. These constraints surely include a geometrical requirement for microtubule proteins to be able to form microtubules; because of the numerous cellular processes involving microtubules, one might expect strong selection against any mutation causing changes in the diameter and shape of this organelle. We can define this constraint more specifically by speaking of a minimum number of contact sites that are necessitated by what we know of the architecture of the microtubule. First, there must be a site where the two monomers interact to form the dimer; this is true regardless of whether the dimers are homogeneous or heterogeneous. Second, there must be another site at which dimers join end-to-end to form the protofilament, and a third where the protofilaments join side-by-side to form the microtubule. One of the latter two may also be the binding site for colchicine. Finally, the dimer has at least one, possibly two, binding sites for guanine nucleotide (9). This site, or sites, may be identical to one of the first three we have mentioned, but it is different from the colchicine-binding site because colchicine and GTP do not compete for binding to microtubules, and, in fact, each stabilizes the other's binding (9). Therefore, there are at least three or four binding sites on the microtubule dimer, each of which imposes certain limitations on the number of permissible mutations in the amino-acid sequences of the tubulins.

In addition to the sites that must be conserved in order to allow a microtubule to be formed, there must also be a certain number of sites on the exterior of the microtubule which are involved in the functioning of the microtubule. Such sites may bind ATPase, flagellar "nexin" (32), microtubule kinase (33, 34), or other enzymes or organizing elements that micro-

† These positions are: in α-tubulin 1, 2, 5, 7, 8, 9, 10, 12, 13, 14, 16, 17, 19, 23, 24, and in β-tubulin 1, 2, 3, 4, 5, 6, 7, 9, 10, 12, 13, 16, 17, 18, 20, 23, 24.

tubules must interact with in order to carry out their functions in nervous tissue or to form the centriole or other microtubule-containing structures (35–37). The necessity for conserving these sites may add to the evolutionary constraints for the tubulins. A fuller understanding of the evolution of the tubulins must await further sequencing of the tubulin species discussed here and also of the tubulins from unicellular organisms such as *Chlamydomonas* and *Tetrahymena*.

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