INVESTIGATION OF TUBULIN FIBERS FORMED DURING MICROTUBULE POLYMERIZATION CYCLES

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Received 29 November 1976
Revised version 8 February 1977

1. Introduction

Microtubules are involved in various cellular functions, such as maintenance of cell-form, formation of the mitotic apparatus, transport of materials in the cell and motility.

Microtubules are polymerization products of tubulin, an asymmetric and dimeric protein. It has been demonstrated that pig brain microtubules can be reassembled 'in vitro' and collected by centrifugation [1]. The assembled material contains both microtubules and flexible fibers, 10 nm thick [2], whose major protein component is tubulin.

In our study, we have examined the microtubules and fibers formed during four cycles of in vitro polymerization. We analyzed the protein content of fibers to determine whether they are aggregates of heterodisperse proteins, or rather possess a fine structure and a precise mode of assembly with specific proteins. Also we investigated the role of GTP in this formation. Furthermore, this analysis led to the modification of the method of Shelanski [1] in order to obtain a higher yield of purified microtubules.

Abbreviations: MES, (2 (*N*-morpholino) ethane sulphonic acid); EGTA, ethyleneglycol-bis (β-amino-ethylether) *N-N'*-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HMW, high molecular weight components; IMW, intermediate molecular weight components; HSS, high speed supernatant

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2. Experimental

2.1. *Preparation of tubulin*

Fresh pig brain was homogenized in an equal volume of reassembly buffer (0.1 M MES adjusted to pH 6.6 with NaOH containing 1 mM EGTA 0.5 mM $MgCl_2$ and 1 mM GTP). Tubulin was prepared by the reassembly–disassembly method of Shelanski et al. [1] (fig.1, Step A, B, C, D). Purified disassembled tubulin was finally suspended in 8 M glycerol-reassembly buffer and stored at −20°C.

Parallel experiments have been carried out with the same buffer, but without GTP (fig.1, Step H, I, K).

2.2. *Preparation of fibers*

Microtubules and fibers are collected by high speed centrifugation and homogenized in 1.0 M NaCl MES buffer, thus the pellet of centrifugation in 1.0 M NaCl buffer (fig.1, Step E) is essentially composed of fibers [2] NaCl was removed from preparations by exhaustive dialysis against MES buffer.

2.3. *Polyacrylamide gel electrophoresis*

Solutions of proteins in 2% SDS were resolved by electrophoresis in polyacrylamide slab-gels (150 × 100 × 1 mm) following procedures described by Laemmli [3]. The slab-gels contained a 6–20% acrylamide-gradient gel. Proteins were stained with Coomassie Blue. Each sample was analyzed in co-migration with standard mixture of proteins of known molecular weight: heavy chains of bovine

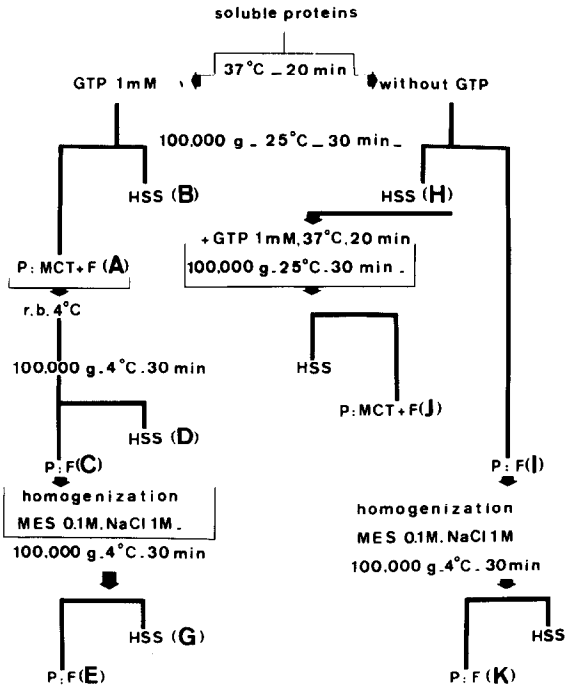

Fig.1. Different steps in the preparation of purified microtubules and tubulin fibers. (P) Pellet, (HSS) high speed supernatant, (r.b.) reassembly buffer, (MCT) microtubules, (F) fibers.

cardiac myosin (200 000) [4], bovine serum albumin (68 000), actin (44 000) [4] light chains of bovine cardiac myosin LC I (27 000) [5] LC 2 (18 000) [4]. All samples were treated with a protease inhibitor phenyl methyl sulfonyl fluoride according to Water et al. [6].

Protein determination was by the method of Lowry et al. [7] using bovine serum albumin as a protein standard.

2.4. *Electron microscopy*

Electron microscopic observations of the sample were made after negative staining. The sample was applied to a 100 mesh grid which was covered with a pioloform F Film [8]. The grid was rinsed with about 10 drops of a 0.1 M KCl solution and stained with one drop of 0.5% uranyl acetate solution in 50% ethanol. The samples were examined in a Philips 300 electron microscope at an accelerating voltage of 80 kV using 50 μm objective aperture.

3. Results

High speed centrifugation after a cycle of polymerization produced a pellet (fig.1, Step A) containing microtubules and fibers (fig.2a). This pellet is homogenized at 4°C in MES buffer, thus the microtubules are depolymerized. After high speed centrifugation at 4°C, the pellet (fig.1, Step C) is composed of fibers and 40 nm rings (fig.2b). Only the rings are dissociated by homogenization in 1.0 M NaCl–MES buffer [2]. Thus, after further centrifugation, the pellet (fig.1, Step E) is composed only of fibers (fig.2c).

To analyse the protein composition the following method was used: the gels of SDS–PAGE are overloaded in order to separate minor proteins which could participate in fiber formation.

After investigation of fibers washed in 1.0 M NaCl–MES buffer, the results showed similar protein composition for each of the cycles of polymerization–depolymerization (first cycle: fig.3, Step E; second cycle: fig.4, Step E; third cycle: fig.3, Step E). The fibers were composed essentially of tubulin and 3 HMW (160 000, 230 000, 300 000) and 2 IMW (64 000, 73 000).

The yields of fibers after the polymerization cycles was: 100 mg/100 g of pig brain for the first cycle, 30 mg/100 g for the second cycle and 10 mg/100 g for the third cycle.

The decrease in the amount of fibers formed was parallel to the decrease in HMW and IMW. There was neither fiber formation nor HMW and IMW after the fourth cycle.

The effect of GTP on fiber formation was then determined. A second cycle was performed in the absence of GTP: the pellet of the first polymerization cycle (Step A) was dissociated in 1.0 M NaCl–MES buffer, fibers were removed by centrifugation and the supernatant was exhaustively dialyzed against reassembly buffer without GTP. The second polymerization cycle without GTP contained no microtubule but fibers were present (fig.1, Step I).

The same amount of proteins was present in the fiber fraction, whether the assembly procedure was carried out in the presence (fig.1, Step E) or absence (fig.1, Step K) of GTP. In either case, the protein distribution on gel electrophoresis was identical (fig.4: Step E, with GTP; Step K, without GTP).

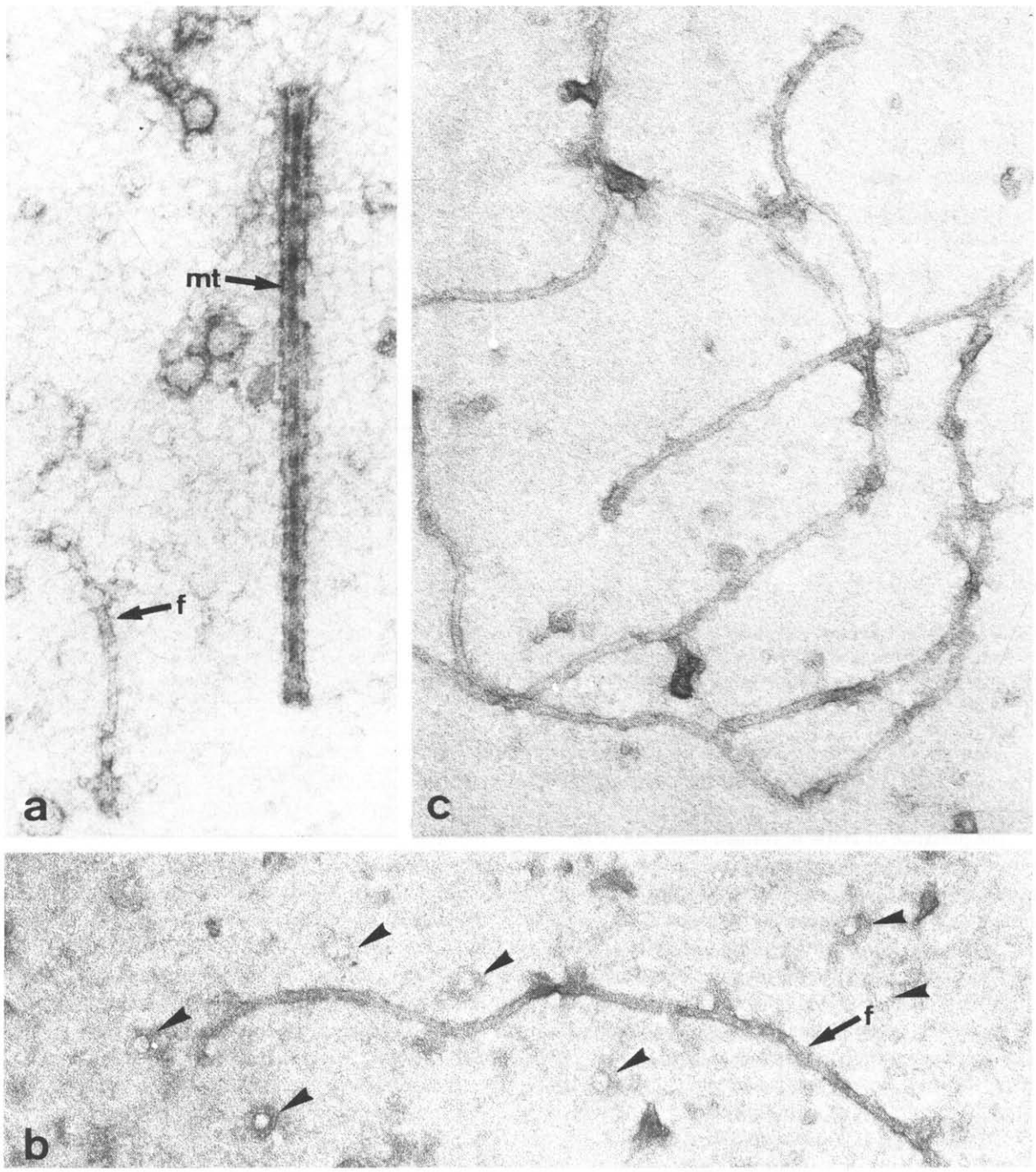

Fig. 2. Electron micrographs of the different steps in the preparation of microtubules and tubulin fibers. (2a) Pellet of centrifugation after a cycle of polymerization showing fibers (f) as well as microtubules (mt). (2b) Pellet of centrifugation after a cycle of depolymerization by low temperature. Ring present beside fibers (arrow heads). Microtubules no longer observed. (2c) Pellet of centrifugation after a cycle of depolymerization by 1.0 M NaCl-buffer. Only fibers are present. Magnification of all figures – × 120 000.

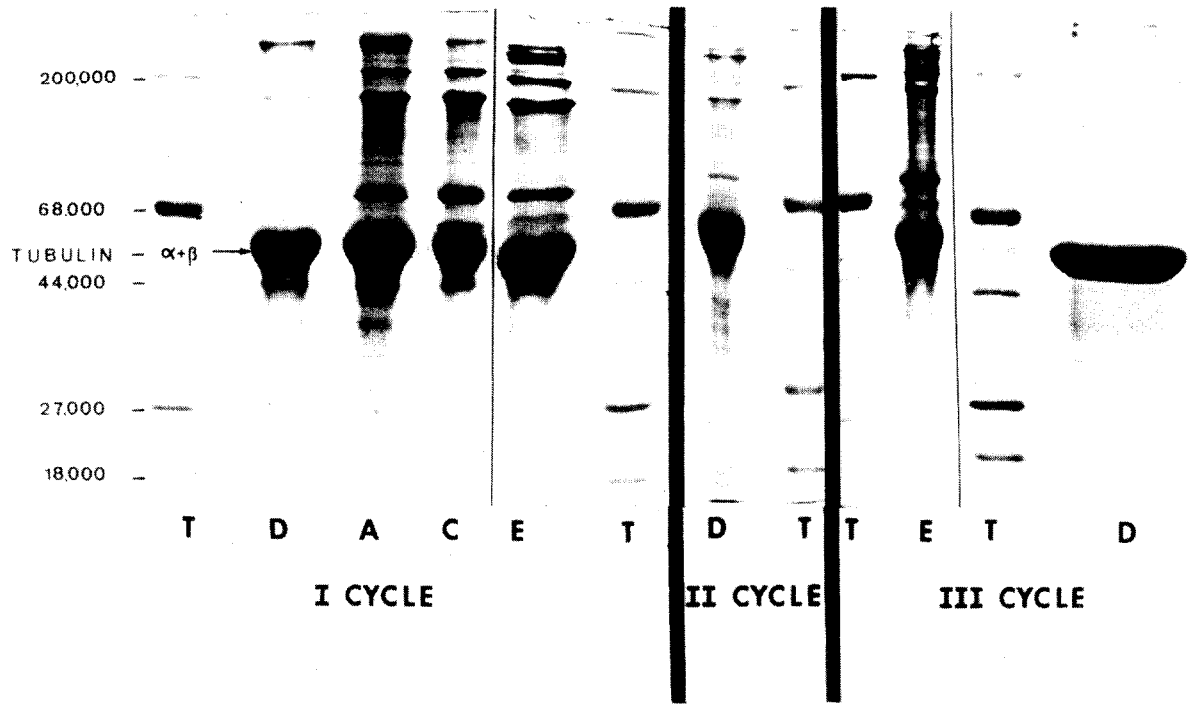

Fig.3. Analysis of microtubule proteins by SDS–PAGE. Study of the pellet and supernatant of high speed centrifugation after each cycle of polymerization. First cycle (I), second cycle (II), third cycle (III). (A) Pellet after reassembly step, (C) pellet and (D) supernatant after a reassembly and disassembly step. (E) Pellet from disassembly step in 1.0 M NaCl buffer, (T) standard marker proteins.

Therefore GTP is not necessary for the formation of fibers.

The presence of HMW in formed fibers led to the investigation of the microtubule protein composition after eliminating insoluble material. Protein composition of microtubules assembled by classical methods [1] is heterogeneous after the first cycle (fig.3, I, Step A). The major protein is tubulin, associated with 2 HMW (300 000 and 330 000) and a wide variety of minor proteins. The results of the protein composition after the second cycle were similar (fig.3, II, Step D). After the third cycle, there is a notable purification of microtubules which were constituted of tubulin (98%) with HMW (300 000 and 330 000) (fig.3, III, Step D). Furthermore, additional bands of lower molecular weight components (28 000 and 30 000) appeared consistently. Microtubule protein composition after the fourth cycle was similar to the precedent cycle.

A comparison of the protein composition of purified microtubules (fig.3, III, Step D) and proteins resolubilized by 1.0 M NaCl (fig.4, Step G) shows that the latter derive from microtubules and rings incompletely dissociated. To obtain a better yield of purified microtubules, we modified the method of Shelanski et al. [1] by passing directly from Step A to Step E and G (fig.1). The microtubule depolymerization was more complete and the amount of soluble proteins was increased. Material obtained at Step G was further dialyzed against reassembly buffer and the microtubules were reconstituted.

4. Discussion

Our study of tubulin fibers formed during in vitro microtubule polymerization cycles showed that, whenever fiber production was significant, there was always the presence of HMW and IMW in the prepara-

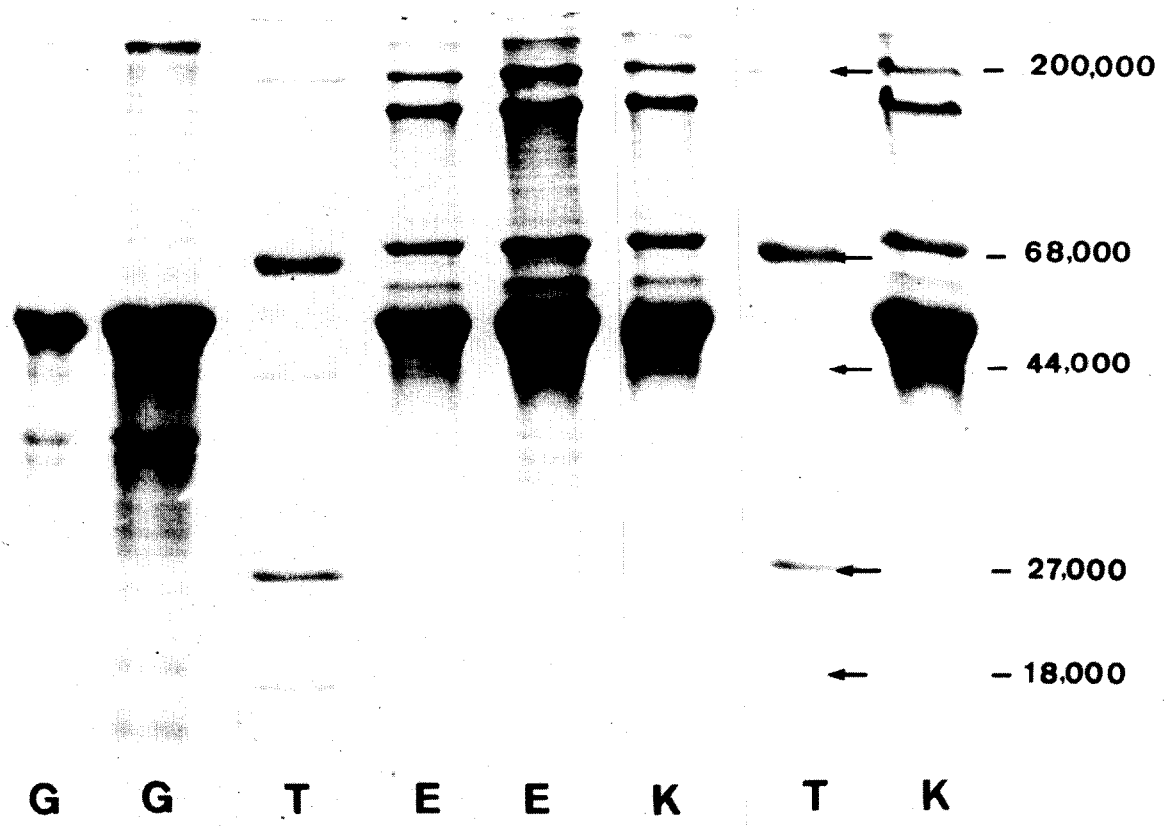

Fig.4. SDS–PAGE of insoluble tubulin preparations obtained from two cycles of assembly–disassembly followed by homogenization in 1.0 M NaCl-buffer. (E) Pellet of the high speed centrifugation, (G) supernatant, (K) preparation without GTP (fig.1), (T) standard marker protein.

tion. Also, the study shows that the process was not influenced by the presence of GTP.

Our report suggests that the presence of specific proteins such as HMW (160 000, 210 000, 300 000) and IMW (64 000, 73 000) is necessary for the formation of fibers. Associated with tubulin monomers α and β, these proteins possess a relatively constant stoichiometry during several polymerization cycles. This association could result from the interactions of these proteins, perhaps basic [9] and acidic tubulin. Furthermore, Sloboda has reported the presence of HMW (330 000 and 300 000) in highly purified microtubules [15]. Future investigation will help establish a relationship, if any, between the HMW 300 000 of the fibers and those of the microtubules.

The literature has reported different protein compositions of microtubules [9–16]. These differences are probably the consequence of the conditions of purification during which the elimination of fibers can be variable. The increase of purification after the third cycle, which we observed, corresponds to removing of all the proteins that have been involved in fiber formation.

A better understanding of the mechanism of fiber formation permits us to obtain highly purified microtubules reconstituted without 'the outer components' which decorate them [17]. These results will lead us to investigate the various roles of protein factors of reassembly.

Therefore, the question is raised, whether the formation of tubulin fibers is a 'pseudo-polymerization' of denatured proteins or whether it has an independent physiological role.

Acknowledgments

Our thanks are due to the electron microscopy investigations of H. F. Hildebrand and the technical assistance of Mlle F. Boutteau and Mme T. N. Dao. The authors wish to express their thanks to Professor M. Dautrevaux for his helpful discussions. This work is subsidised by a financial aid of RCP No. 386 Dr D. Pantaloni and ERA No. 32 of Professor G. Biserte.

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