Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 386-394

INCORPORATION OF GDP-TUBULIN DURING ELONGATION OF MICROTUBULES IN VITRO

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Received July 22, 1985

SUMMARY: Removal of GDP from tubulin E-site is not obligatory for the in vitro assembly of microtubule protein in 0.5 mM GMPPCP. This assembly, which is significantly enhanced by glycerol, produces microtubules of normal morphology and with normal composition of microtubule-associated proteins (MAPs). [^{3}H]-GDP initially present at the E-site is shown to be incorporated directly into microtubules during assembly; this incorporation, maximally 60% of the assembled polymer, is dependent upon MAPs. These results are consistent with oligomeric species composed principally of GDP-tubulin plus MAPs, being incorporated directly into microtubules. The finding that stoichiometric GTP-tubulin formation is not an essential prerequisite for microtubule assembly may have important implications for the energetics of microtubule formation. © 1985 Academic Press, Inc.

Tubulin αβ heterodimer contains two non-identical guanine nucleotide binding sites (1,2). The non-exchangeable (N) site contains GTP but the exchangeable (E) site can accommodate a number of analogues in addition to GTP or GDP (3,4). The general view of in vitro assembly is of stringent requirement for GTP (or analogue) at the tubulin E-site (5). Here we examine the fate of tubulin E-site GDP during assembly with GMPPCP. Although the assembly-induced GTP-ase activity is a property of the E-site (6,7), it is known that GMPPNP and GMPPCP, the non-hydrolysable analogues of GTP, can

ABBREVIATIONS : GMPPCP, 5′-guanylyl methylene diphosphate; GMPPNP, 5′ guanylyl imidodiphosphate; GTP, guanosine-5′-triphosphate; GDP, guanosine-5′-diphosphate; GMP, guanosine-5′-monophosphate; MT-protein, microtubule protein; MAPs, microtubule-associated proteins; HPLC, high performance liquid chromatography; EGTA, ethylene glycol bis(-aminoethylether)-N,N,N′,N′-tetra-acetic acid; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis, MES, 2(N-morpholino) ethane sulphonic acid.
GDP-tubulin : tubulin with GDP at the exchangeable nucleotide binding site.

0006-291X/85 $1.50
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promote assembly of tubulin under appropriate conditions (8,9,10). The critical concentration for analogue-induced MT-protein assembly is similar to that with GTP (11), but the resultant microtubules are more cold stable (12). The K_d of these analogues has been estimated at $3x10^{-6}$M (13), approximately 100-fold higher than that of GTP or GDP (14). The extent of analogue incorporation into microtubules can depend upon the success in removing E-site GDP by treatment with charcoal (10) or alkaline phosphatase (15). Added GDP is strongly inhibitory to GMPPCP-induced assembly (10).

The fate of E-site GDP in GTP-induced MT-protein assembly is complicated by GTP-ase (16) and transphosphorylase (17) activities which co-purify with MT-protein. These complications are avoided by using GMPPCP rather than GTP. The importance of direct GDP-tubulin incorporation can thus be assessed with GMPPCP-induced assembly in the absence of free GDP and GTP, using MT-protein in which the E-site nucleotide is known to be exclusively GDP.

We describe in this report the assembly of MT-protein in 0.5 mM GMPPCP in the presence of glycerol. Analysis of the guanine nucleotide content shows that 30-60 % of the [^{3}H]-GDP label present at the E-site of assembled microtubules derives from [^{3}H]-GDP-tubulin incorporated directly during the assembly reaction. These results provide further confirmation of the incorporation of oligomeric species in microtubule elongation in vitro (18,27).

The complete exchange of nucleotide-triphosphate-tubulin into the oligomeric fragments is clearly not a prerequisite for this assembly with microtubules. We suggest as a minimum mechanism that the occurrence of GTP-tubulin in a terminal position is sufficient to provide the assembly of a protofilament fragment.

MATERIALS AND METHODS

[^{3}H]-GTP was purchased from Amersham (U.K.), GMPPCP from Boehringer Mannheim, tetrabutylammonium sulphate and methyl alcohol from Aldrich, and alkaline phosphatase type VII from Sigma. All other reagents are as described in (19).

MT-protein was purified from bovine brain using a cycling method as in (19), and stored at -70°C in 50 % glycerol. Before each experiment the protein was assembled in 0.5mM GTP, or in 0.1 mM GTP + 5 Ci [^{3}H]-GTP / ml, and the microtubules harvested by centrifugation (100,000g 40 min.). The pellets were resuspended in MEM100 (100 mM MES, 0.1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.5), disassembled at 4°C for 30 minutes, and clarified by centrifugation (80,000 g, 30 min.). Free nucleotide was removed from the protein by Sephadex G-25 gel filtration on a 0.9 x 5 cm column at 4° C.

Microtubule polymerisation at 37°C was monitored by turbidity, and the digitised data was collected and analysed as the sum of exponential functions as described previously (18). The protein and nucleotide content of the assembled microtubules were determined by centrifugation of 175 µl aliquot of the reaction mixture in a prewarmed Beckman A100 rotor (140,000 g,6 min.) The supernatant was removed and the pellet resuspended in 175 µl of MEM100. Nucleotide was released from the tubulin by 2.5% perchloric acid precipitation and centrifugation (10 min. bench centrifuge), the supernatant was neutralised with KOH and taken for HPLC analysis or counting. Guanine nucleotides were separated as in (20) by reverse phase HPLC using an Arksil C-18 column with a running buffer containing 1.3% KH_2PO_4, 0.3% tetra-butylammonium, and 5% methyl alcohol, pH 6.5. The detector (Waters model 450) was set at 254 nm and nucleotide concentrations determined from the peak areas or scintillation counting of the relevent fractions.

Protein analysis was by SDS electrophoresis with 7.5 % acrylamide slab gels, stained with Coomassie blue,and densitometry as in (18). Protein concentrations were determined spectrophotometrically at 276 nm with A (1cm) = 1.12 per mg/ml of MT-protein.

RESULTS : The assembly of Sephadex G-25 treated MT-protein in the presence of 0.5 mM GMPPCP (Fig.1a) is characterised by a longer lag phase and a reduced assembly amplitude compared with assembly in GTP. However, in the presence of glycerol, this lag is reduced and elongation is rapid (Fig.1 b,c,d), the plateau achieved is 80 % of that in GTP. (This acceleration is opposite to the effect of glycerol for assembly in 0.5 mM GTP, (19)). Analysis of the assembly trace in 1M glycerol, (Fig.1b) (18), reveals biphasic kinetics as for GTP induced assembly (19). The rate for the fast phase in GMPPCP (0.01 +/-0.005 sec^{-1}) is half that in GTP under the same buffer conditions.

The most striking result is that efficient GMPPCP-induced assembly occurs without prior removal of E-site GDP. The authenticity of this assembly is established as follows:

(i) The purity of the GMPPCP was determined by HPLC which showed the presence of a single peak at a position between that of GDP and GTP and accounted for > 99% of the absorbance at 254 nm. At higher

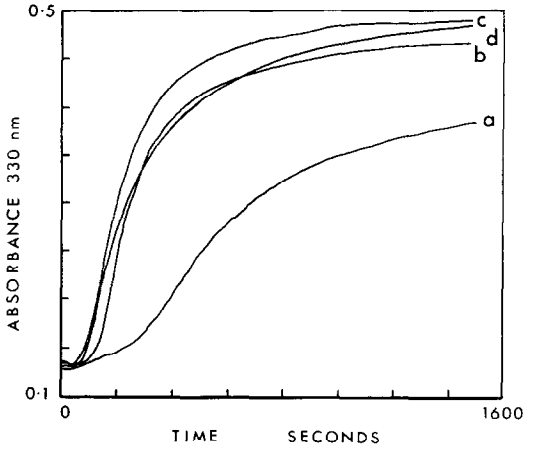

FIG.1 Effect of glycerol on the assembly of MT-protein in the presence of 0.5 mM GMPPCP. MT-protein, containing stoichiometric exchangeable GDP was assembled at 2.2 mg/ml in MEM100 buffer containing varying amounts of glycerol. Turbidity was monitored at 330 nm with a 1 cm pathlength cell: assembly was initiated by adding the reaction mixture from ice to the warm cell (37°C). Glycerol concentrations were as follows: 0 M (a) ; 1M (b) ; 2M (c) ; 4M (d) .

resolution a smaller peak (0.87%) could be identified at a position corresponding to GMP + guanosine. Treatment of a solution of GMPPCP (0.5 mM) with alkaline phosphatase (40 units/ ml) to hydrolyse di-and tri-phosphate, showed by an increase in this peak by 0.6 % of the total representing a total contaminating (GDP + GTP) of 3 μM.

(ii) The MT-protein does not undergo assembly in the absence of added nucleotide (GTP or GMPPCP). Guanine nucleotide analysis of [^{3}H] label incorporated in the preparative cycle (see methods) and released

TABLE 1. HPLC analysis of [^{3}H]-guanine nucleotides

SAMPLE	PERCENTAGE OF [^{3}H] COUNTS		
	GTP	GDP	GMP
(a) G-25 MT-Protein	2	91	7
(b) SUPERNATANT	0	56	11
(c) PELLET	0	33	0

(a) MT-protein after Sephadex G-25 gel filtration.
(b) the supernatant fraction after centrifugation of this MT-protein assembled , 0.5 mM with GMPPCP in MEM100 + 3M glycerol (as descibed in Fig.3).
(c) the pellet fraction resuspended in the original volume of buffer.

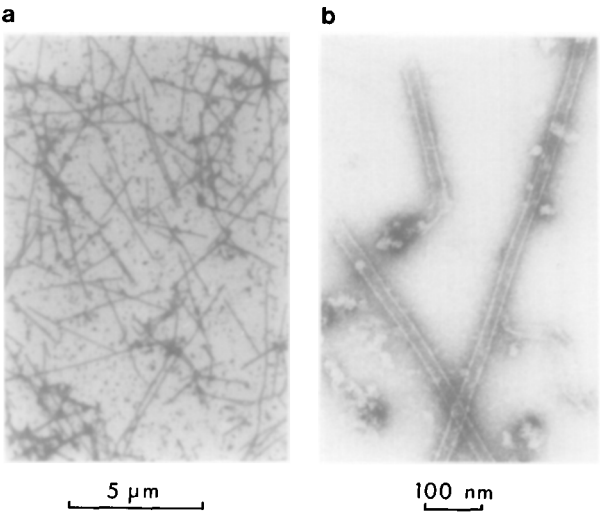

FIG. 2 Electron microscopy of GMPPCP-induced microtubules assembled in MEM100 buffer, at (a) low and (b) high magnification. The assembled protein was absorbed onto carbon coated grids and stained with 1% uranyl acetate before viewing with a Philips 301 electron microscope.

from the protein by perchloric acid (table 1) shows that less than 2% of the exchangeable nucleotide (E-site) was GTP.

(iii) Electron microscopy of the assembly product in aqueous and glycerol buffers shows that the GMPPCP induced microtubules are of normal morpholgy (Fig.2b) and have an average length of less than 5 μm (Fig.2a). These microtubules are shown by SDS-PAGE of the pelleted material to contain >90 % of the high molecular weight (HMW) MAPs.

To confirm that the analogue itself was incorporated, microtubules assembled with 0.2 mM GMPPCP in 3M glycerol were pelleted in the airfuge, and the nucleotide content was analysed by HPLC. Non-specific trapping of nucleotide within the pellet was estimated by adding calibrated amounts of GMP. This pelleted protein contained 60% GMPPCP and 40% GDP relative to the N-site GTP as 100%. The source of the GDP in the pellet was determined by using [^{3}H]-GDP-tubulin in the assembly mixture. Results are presented in Table 1. The GDP in the pellet derives entirely from [^{3}H] E-site GDP present in the MT-protein before assembly. The proportion of [^{3}H]-GDP-tubulin present in the

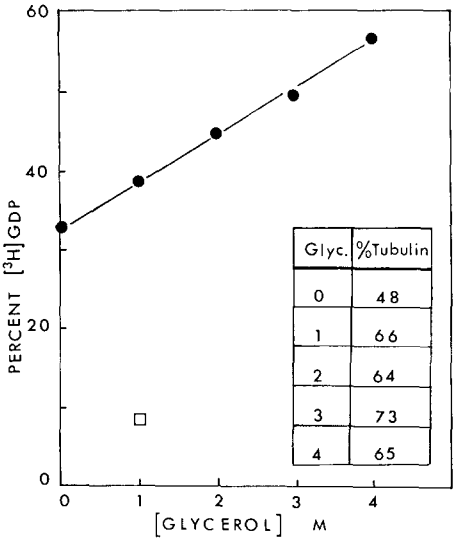

FIG. 3 The mole percentage of [^{3}H]-GDP incorporated into microtubules assembled in 0.5 mM GMPPCP as a function of glycerol concentration. MT-protein (1.5 mg/ml) containing stoichiometric [^{3}H]-GDP was assembled as described in Fig.1. Samples were removed at 25 min. and the microtubules rapidly pelleted (Beckman airfuge 140,000 g 6 min.) in a pre-warmed rotor. The pellets were resuspended in MEM100 and both the supernatent and pellet fractions counted. Samples were also taken for SDS-PAGE; the percentage of tubulin in each pellet was calculated by densitometry of the tubulin bands (see table insert). The percentage of counts in the pellet divided by the fraction of tubulin in the pellet gives the percentage of E-site occupied by [^{3}H]-GDP in the microtubules (●). As a control MT-protein was assembled in 0.5 mM GTP in MEM100 + 1M glycerol, and the percentage of [^{3}H] nucleotide incorporated calculated in a similar manner (□).

microtubules was determined as a funtion of glycerol concentration (Fig.3). There is an approximately linear increase in [^{3}H]-GDP content, ranging from 30% in the absence of glycerol to 60% in 4M glycerol. For comparison the experiment was repeated for MT-protein with 0.5 mM GTP in 1M glycerol. 8% label was observed in the pellet; this presumably includes incorporation from direct and indirect (i.e. transphosphorylation-mediated) sources.

DISCUSSION : GMPPCP promotes the assembly of normal microtubules from MT-protein containing >90 % E-site GDP. Analogue-induced assembly has previously been shown to be very sensitive to added free GDP (10). Under our conditions any free GDP remaining after gel filtration is

insufficient to inhibit assembly, although it may preclude attainment of the full assembly amplitude. The rapid assembly in solutions containing glycerol indicates efficient nucleation; this is consistent with the relatively short microtubles (Fig. 2). Pulse-chase experiments have demonstrated that the non-hydrolysable analogues are chased from microtubules only extremely slowly at steady state (12). We conclude that a mechanism exists for direct GDP-tubulin incorporation into normal microtubules.

GDP has been shown to be more potent an inhibitor of nucleation than of elongation (11). The rapid assembly observed in glycerol implies that generation of the required prenucleating species is fast. The proportion of [^{3}H]-GDP in the assembled microtubules is similar to that of tubulin dimer present as oligomeric species in MT-protein (21). It is known that nucleotide in oligomeric rings is virtually non-exchangeable with free nucleotide (22); our results indicate that the fragments deriving from these rings incorporate into microtubules with retention of most of their GDP-tubulin. An alternative elongation mechanism might involve the addition of solitary GDP-tubulin dimer; however, this has been shown to be extremely slow (23) and highly unfavourable (24). By contrast, the assembly rates observed here are comparable with those for MT-protein in GTP. We have recently reported pyrophosphate-induced assembly (in the absence of GTP) of tubulin and MT-protein, depleted in E-site GDP by alkaline phosphatase pretreatment; this produces rapid assembly kinetics and again shows the incorporation of [^{3}H]-GDP tubulin [20]. Previous data involving the assembly of GDP-tubulin are somewhat controversial (25). A slow assembly of tubulin dimer in 0.7 mM GDP (25) and some assembly of GDP containing MT-protein (11,22) have been reported. A critical review (26) concludes that more precise nucleotide analysis is required. This has been met here by HPLC criteria.

As a minimal requirement we suggest that the addition of an assembly competent fragment with appropriate polarity may depend on the formation of a "sticky end" through generation of GTP- or analogue-containing terminal tubulin dimer and that this fragment may then incorporate efficiently, irrespective of the remaining subunits. A possible mechanism for this could involve synchronous ring opening and limited terminal dimer exchange; fragment addition (18,27) would then follow. This mechanism is also consistent with observations of protofilament-like oligomers in close proximity to microtubule ends (28). The action of glycerol in the enhancement of assembly and GDP-tubulin incorporation suggests stabilisation of intermediate oligomeric species by promotion of tubulin-tubulin interactions (cf. 29).

These results could have important implications for GTP utilisation in MT-protein assembly. With excess GTP, the assembly species presumably contain predominantly GTP at the E-site. With minimal GTP, a different situation may exist; the addition of GDP-tubulin containing oligomers could occur by means of 'activated' GTP-tubulin ends. In vivo, pre-formed oligomeric complexes of tubulin with MAPs (and possibly other cytoskeletal components) could be activated for microtubule assembly with a minimal requirement for guanine nucleotide triphosphate.

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