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TUBULIN POLYMERISATION IN THE PRESENCE OF GMP-PCP

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SUMMARY: Microtubules induced by the binding of GTP or of a non-hydrolysable analog of GTP onto the exchangeable site of tubulin appear very similar according to electron microscopy and polymerisation kinetics criteria. However,we show here that the exchangeable sites or "E" sites of the tubulin subunits remain available for nucleotide exchange inside the GMP-PCP-microtubules contrary to the "E" sites inside the GDP-microtubules. Moreover,under specific conditions,GMP-PCP induces the polymerisation of tubulin into a bidimensional,pseudocrystallin structure. Such a "crystallisation" is inhibited by GTP and GDP.

INTRODUCTION: Microtubule assembly in vitro requires the binding of GTP or of a non-hydrolysable analog of GTP onto the exchangeable nucleotide site or "E" site of tubulin[1-6]. When microtubules are assembled in the presence of GTP, the GTP is hydrolysed during the polymerisation process[2,5],leaving a non-exchangeable GDP onto the "E" sites of the tubulin subunits inside the microtubules[2,6]. The fact that microtubule formation could be induced as well in the presence of the non- hydrolysable analogs of GTP,guanylyl-β,γ-methylene diphosphate (GMP-PCP) and guanylyl imido-diphosphate (GMP-PNP),suggested that GTP hydrolysis was not required in the assembly process. Furthermore it was reported that,according to several criteria including electron microscopy and polymerisation kinetics[4,8],the microtubules induced by GTP and the non-hydrolysable GTP analogs were very similar. However it has also been reported that GMP-PNP-microtubules do not display evidence for a rapid equilibrium between polymers and subunits contrary to GDP-microtubules,suggesting that GTP hydrolysis would promote a more readily depolymerisable state of tubulin[9].

We show here that microtubules induced in the presence of GMP-PCP and GTP differ also by other criteria,especially the "E" sites of the tubulin subunits inside the GMP-PCP-microtubules remain available for nucleotide exchange contrary to the "E" sites inside the GDP-microtubules. Moreover,we show that,

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under specific conditions,GMP-PCP induces the polymerisation of tubulin into a bidimensional,pseudocrystallin structure and that this"crystallisation"is inhibited by GTP and GDP.

MATERIALS AND METHODS: Tubulin was isolated from pig brain homogenates by three cycles of polymerisation and depolymerisation(10). The assembly buffer used for purification and assembly experiments contained 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) adjusted to pH 6.6 with NaOH/1mM ethylene bis(oxyethylene nitrilo)tetraacetic acid (EGTA)/0.5mM $MgCl_2$.

Tubulin was purified further and separated from the MAPs on a phosphocellulose column as described elsewhere(11).

Turbidity measurements were made at 350nm on a Beckman Acta V spectrophotometer in cells of 0.5cm light path.The polymerisation reaction was started by a temperature jump from 4° to 37°,which required a half-time of 4 sec.

The liberation of[^{32}P]labeled inorganic phosphate was measured according to the procedure of Nishizuka et al.(12). Before experimentation,free nucleotides were removed by passage on a G25 sephadex column.

Protein concentration was determined by the method of Lowry et al.(13) with a correction of 10% for the color difference between tubulin and bovine serum albumin(14).

RESULTS: Nucleotide exchange inside the GDP- and the GMP-PCP-microtubules

The experiments have been performed on phosphocellulose-purified tubulin preparations.After passage on a G25 sephadex column,we found,like others (2,15),that the tubulin dimer still contained one GTP on its non-exchangeable or "N" site and one GTP on its "E" site. The "E"site GTP was converted into GDP through a polymerisation cycle at 37° in a buffer at pH 6.6 containing 0.05M MES, 0.5mM EGTA,10mM Mg^{++} and 30% glycerol (RBMG).After this polymerisation cycle, the tubulin preparation (23µM) was unable to initiate a second polymerisation cycle without adding either GTP or GMP-PCP. The tubulin preparation was then separated into two parts. On one pool,the polymerisation reaction was started by the addition of 3mM GMP-PCP and after 30 min at 37°,that is,after an apparent equilibrium had been reached,300µM [^{3}H,^{32}P]-labeled GTP was added. The labeled GTP addition did not change at all the quantity of polymerised material according to turbidity measurement. Also,the microtubule solution was observed under the electron microscope before and right after the addition of labeled GTP. The microtubules apparently did not break down and reform after the GTP addition. We suppose then that,like in the case of the GMP-PNP-microtubules(9),the GMP-PCP-microtubules do not display evidence for a rapid equilibrium between polymers and

subunits. At time zero and 25 min at 37°after the GTP addition,microtubules were sedimented through a 25% sucrose cushion for 20 min at 160,000g. The sedimentation at time zero gave an estimate of the radioactivity contamination. After substracting the blank,16μM protein and 14μM [^{3}H]-labeled GDP were found in the microtubule pellet,that is a "E" site GTP to tubulinstoichiometry very close to 1:1. It appeared then that the [^{3}H,^{32}P] -labeled GTP had chased the GMP-PCP from the microtubule "E" site. Also,nucleotide analysis by thin layer chromatography clearly demonstrated that the GMP-PCP bound to the microtubules had been chased by GTP.

On the other tubulin pool,a control experiment was runned in order to confirm the already known observation that the nucleotide on the "E" site inside the microtubules polymerised solely in the presence of GTP is unexchangeable(2-5). Microtubules were polymerised in the presence of [^{3}H,^{32}P] -labeled GTP at 37°for 30 min until an apparent equilibrium was reached. At that time,one part of the sample was centrifuged while to the other part was added 2mM unlabeled GTP. 16μM tubulin and 12μM [^{3}H]-labeled GDP were found in the microtubule pellet.After chasing for 25 min at 37°with the excess unlabeled GTP,the microtubules still bound 0.65 mole of [^{3}H]-labeled GDP per mole of tubulin.

Inorganic phosphate release during the chase of GMP-PCP by GTP.

The time course of inorganic phosphate release was followed during the chase of GMP-PCP by the [^{3}H,^{32}P]-labeled GTP. The kinetics shown in fig.1 appear clearly biphasic. There is a rapid initial inorganic phosphate release (3μM min^{-1}) after which the GTPase activity slows down continuously until it reaches a steady state (1.5μM min^{-1}). This time course of GTP hydrolysis ressembles very much that obtained when the microtubule formation is initiated directly with GTP(14). The steady state rate of GTP hydrolysis is attained about 20 min after the addition of labeled GTP and should be due to the GTPase activity at the microtubule ends(7). Notice that the final,linear portion of the curve extrapolates at about 20μM which is of the order of magnitude of the [^{3}H]-labeled GDP concentration bound to microtubules. This observation indicates that there must be only one GTP hydrolysed per tubulin

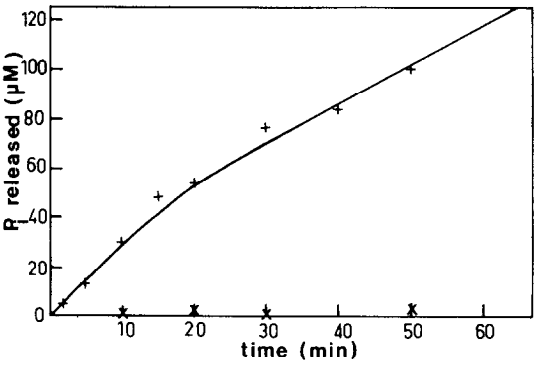

Fig.1: Time course of inorganic phosphate release following the addition of 300μM [^{3}H, ^{32}P]-labeled GTP to a preparation of GMP-PCP-microtubules at 37°. The crosses show that no GTP hydrolysis is occurring when the tubulin polymerisation is inhibited by 30μM vinblastine.[17] The tubulin concentration was 23μM.

dimer inside the microtubules. In other words,once a GTP has chased a GMP-PCP from a microtubule "E" site and has been hydrolysed,then the GDP on that site becomes unexchangeable.

"Crystallisation" of tubulin in the presence of GMP-PCP.

The experiments reported in the previous sections were performed at relatively low total tubulin concentration (23μM) and,under these conditions,in RBMG buffer, microtubules were observed under the electron microscope when the polymerisation reaction was induced with GMP-PCP. However,at higher tubulin concentration (35μM),under the same buffer and temperature conditions (RBMG,37°),microtubules were not anymore formed. Rather,very long and large bidimensional structures such as that presented in fig.2 were observed. Some of these structures extend up to 5u in width and more than 40μ in length. These preliminary observations do not show any regular structures analogous to the large sheets observed in the presence of Zn ions[16]. These structures could be amorphous or polycrystallin with small patches in which the subunits would be arranged into protofilaments like in microtubules. More work is needed to elucidate the structure of these large bidimensional polymerised films. When GTP is added,the structures first fall apart and eventually microtubules form. There is no GTP hydrolysis during the breaking down of the films but,as expected,GTP hydrolysis accompanies the microtubule formation. GDP addition also destabilises the structures but,in that case,microtubules do not form afterwards.

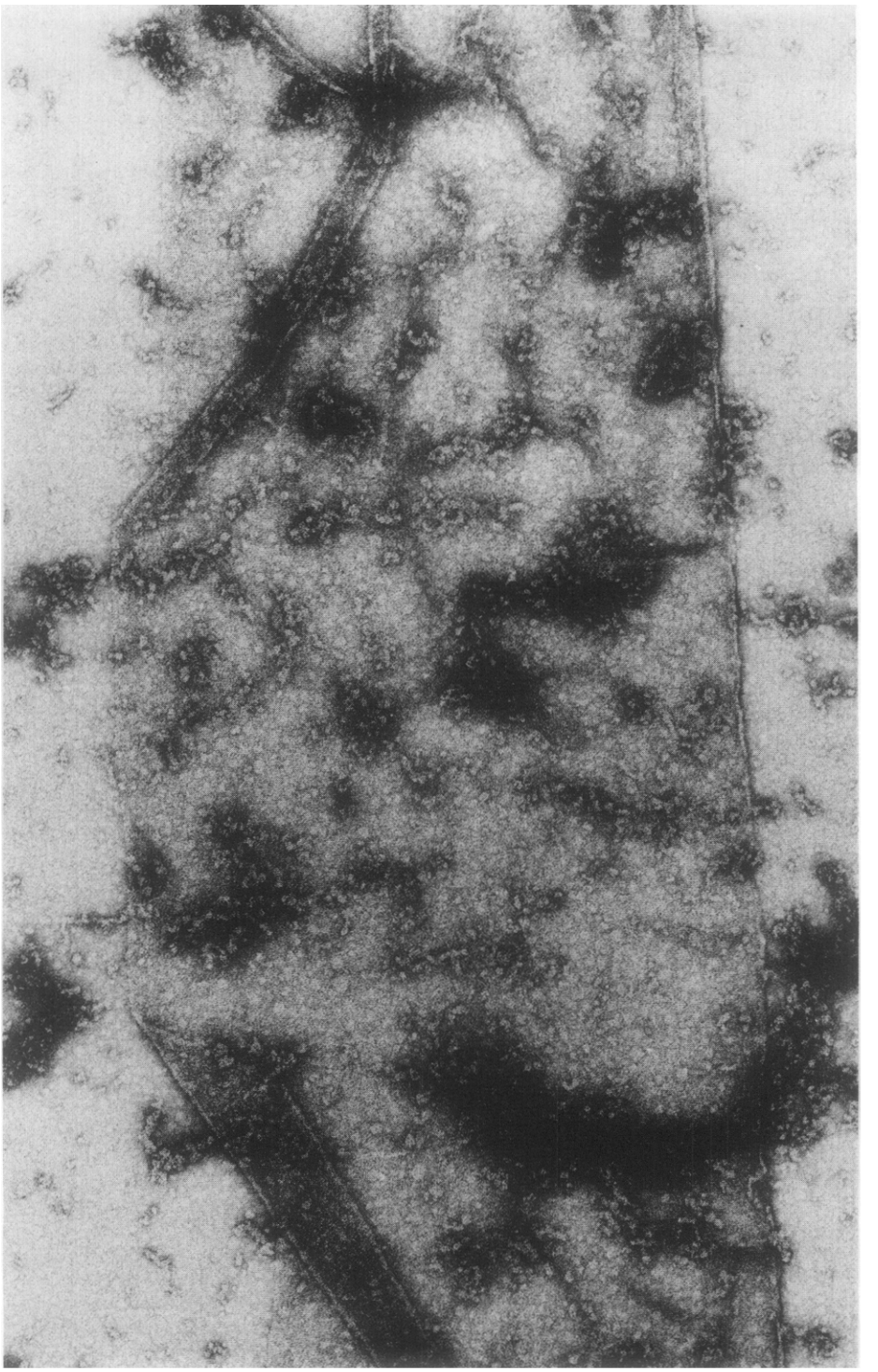

Fig.2:Electron micrograph of a giant bidimensional film induced by the addition of 3mM GMP-PCP to a phosphocellulose purified tubulin preparation (35µM) in RBMG buffer. X 80,000.

DISCUSSION: The observations reported here bring some new light onto the function of the guanine nucleotides in microtubule assembly. The destabilisation by GTP of the giant GMP-PCP polymers occurs in the absence of GTP hydrolysis,suggesting that the state of tubulin inside the GMP-PCP polymers is not in a conformation active in GTP hydrolysis. The role of GTP in inhibiting the formation of aberrant tubulin polymers appears therefore to be conformational.

At relatively low tubulin concentration,GMP-PCP induces the polymerisation of tubulin into apparently normal microtubules. However,the nucleotides on the "E" sites of the GMP-PCP-microtubules remain exchangeable contrary to the GDP on the "E" sites of the microtubules polymerised with GTP. We have shown here that the binding of a GTP molecule onto a "E" site inside a microtubule resulted into hydrolysis of the GTP and then blocking of the site. This suggests that the state of tubulin inside the microtubules exists in a conformation active in GTP hydrolysis and that the role of the GTP hydrolysis is to promote the blocking of the nucleotide sites. Thus, the apparent observation that the tubulin subunits inside the microtubules are lacking GTPase activity[14] is attributable to the fact that the "E" sites inside the microtubules are not available for nucleotide exchange.

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REFERENCES:

1. Weisenberg,R.C. (1972) Science,177,1104-1105.
2. Jacobs,M.,Smith,H.& Taylor,E.W. (1974) J.Molec.Biol.,89,455-468.
3. Kobayashi,T. (1975) J.Biochem.,77,1193-1197.
4. Arai,T.& Kaziro,Y.(1976) Biochem.Biophys.Res.Commun.,69,369-376.
5. Weisenberg,R.C.,Deery,W.J.& Dickinson,P. (1976) Biochemistry,15,4248-4254.
6. Lockwood,A.H.,Penningroth,S.M.& Kirschner,M.W. (1975) Fedn.Proc.,34,540.
7. David-Pfeuty,T.,Laporte,J.& Pantaloni,D. (1978) Nature,272,282-284.
8. Penningroth,S.M.& Kirschner,M.W. (1977) J.Molec.Biol.,115,643-673.
9. Weisenberg,R.C.& Deery,W.J. (1976) Nature,263,792.
10. Shelansky,M.L.,Gaskin,F.& Cantor,C.R. (1973) Proc.Natl.Acad.Sci.U.S.A.,72,765.
11. Weingarten,M.D.,Lockwood,A.H.,Hwo,S& Kirschner,M.W. (1975) Proc.Natl.Acad.Sci. U.S.A.,72,1858-1862.
12. Nishizuka,Y.,Lipmann,F& Lucas-Lenard,J. (1968) in Methods in Enzymology,eds. Grossman,L.& Moldave,K. (Academic Press,New York),vol.12B,pp.708-721.
13. Lowry,O.H.,Rosebrough,N.W.,Farr,A.L.&Randall,R.J. (1951) J.Biol.Chem.,193,265.
14. David-Pfeuty,T.,Erickson,H.P.& Pantaloni,D. (1977) Proc.Natl.Acad.Sci.U.S.A. 74,5372-5376.
15. Huitorel,P. (1979) 3rd cycle Thesis,Paris.
16. Larsson,H.,Wallin,H.,Edstrom,A. (1976) Exp.Cell Res.,100,104-110.
17. Marantz,R.& Shelanski,M.L. (1970) J.Cell Biol.,44,234.