

Control of nucleation in microtubule self-assembly

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The inhibition of the rate and amplitude of assembly of microtubule protein at low GTP concentration is shown by measurement of microtubule length distributions to be due to the suppression of microtubule nucleation. This inhibitory effect is enhanced by GDP added before assembly, but can be overcome by a number of molecules such as pyrophosphate or ADP. The selective inhibition of nucleation by GDP *in vitro*, which occurs in addition to inhibition of elongation, could provide a mechanism for the control of spontaneous microtubule nucleation *in vivo*.

Microtubule Assembly Nucleation GTP GDP Pyrophosphate

1. INTRODUCTION

The guanine nucleotides GTP and GDP exert complex effects in the assembly of microtubules [1–5]. In aqueous buffer, the assembly of microtubule protein (i.e. tubulin dimer plus the microtubule associated proteins, MAPs) is inhibited by GDP. GTP stimulates assembly, with consequent hydrolysis to GDP. Non-hydrolysable analogues of GTP support assembly; GDP-tubulin can be incorporated directly into assembling microtubules as MAP-containing oligomeric species [6] and nucleotide-depleted tubulin dimer can assemble with MAPs into normal microtubules in the presence of pyrophosphate ion [7].

Particular interest surrounds the opposing actions of GDP and GTP. The inhibitory effects of GDP during elongation can be accounted for by the equilibrium of GDP-tubulin and GTP-tubulin [2–5]. Spontaneous changes in length distributions at steady state of microtubules (in the absence of MAPs or glycerol) have been interpreted in terms of a 'dynamic instability' deriving from the different kinetic properties of GTP-tubulin and GDP-

tubulin [8,9], and extensive modelling of this system has been performed [10]. Inhibitory effects due to non-productive binding of GTP-tubulin and GDP-tubulin have also been proposed to account for anomalous kinetic properties [11,12].

In the course of systematic investigations of a number of factors on the properties of microtubule protein in aqueous buffers [2–7,13,14], we have studied the effect of GTP concentration on the assembly characteristics. Assembly rates are proportional to the product of the intrinsic elongation rate constant and the microtubule number concentration, which can be assessed by electron microscopy of microtubule length distributions.

We show here that microtubule nucleation is markedly impaired as [GTP] is reduced towards a stoichiometric ratio with [tubulin]. The assembly rate is slower, the amplitude is decreased and the length distribution is several-fold longer. Efficient nucleation can be restored at low [GTP] conditions by addition of a sub-millimolar concentration of one of a number of compounds. Conversely, nucleation is inhibited by the addition of GDP prior to assembly. These results strongly implicate GDP as being responsible for the observed suppression of microtubule nucleation, with marked effects on the kinetics of GTP-induced self-

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assembly of microtubule protein *in vitro*. By extension, GDP appears potentially capable of exerting control of spontaneous assembly of microtubules *in vivo*.

2. MATERIALS AND METHODS

Preparation of microtubule protein from bovine brain was as described [13]; all experiments were performed in standard buffer (0.1 M Mes, 0.1 mM EGTA, 0.5 mM Mg, pH 6.5). Microtubule protein was prepared free of additional nucleotide by passage of cycled MT-protein over Sephadex G-25; this material contains >95% GDP at the exchangeable nucleotide binding (E) site [7]. GTP was then added to this preparation to give the appropriate concentrations prior to assembly. Assembly was monitored continuously as in [13]; the maximal initial rate was determined by numerical differentiation of the assembly curve. Microtubule seeds were prepared from assembled MT-protein by passage through a 26-gauge syringe needle, to give fragments 1–2 μm in length. Length distributions were measured (using a Graphics pad and a PDP 11/23 computer) on micrographs of negatively stained microtubule samples fixed with 0.25% glutaraldehyde at [protein] = 0.03 mg/ml.

AMPPNP was obtained from Boehringer-

Mannheim; ADP, ATP, GDP, GTP, sodium pyrophosphate and tripolyphosphate were obtained from Sigma, and used as supplied.

3. RESULTS

The assembly of MT-protein with [GTP] = 15 μM (fig.1a) shows a pronounced lag phase, and the initial rate and amplitude of assembly are reduced relative to the control. Inclusion of 0.5 mM pyrophosphate largely reverses this effect. The dependence of initial rate on [GTP] increases about 3-fold up to [GTP] = 2 mM, above which there is inhibition (cf. [2]). The dependence on [pyrophosphate] with [GTP] = 15 μM also shows a similar stimulation up to 3 mM, followed by inhibition.

To examine whether pyrophosphate alters the intrinsic elongation rate constant, the elongation process for seeded assembly with [GTP] = 15 μM (fig.1b) is seen to be unaffected in rate or amplitude by the presence of 0.5 mM pyrophosphate. In confirmation of this, the rate of self-assembly of microtubule protein with [GTP] = 15 μM showed no effect when pyrophosphate (0.5 mM) was added after the nucleation phase, i.e. after maximum rate was attained.

Electron micrographs show that microtubules

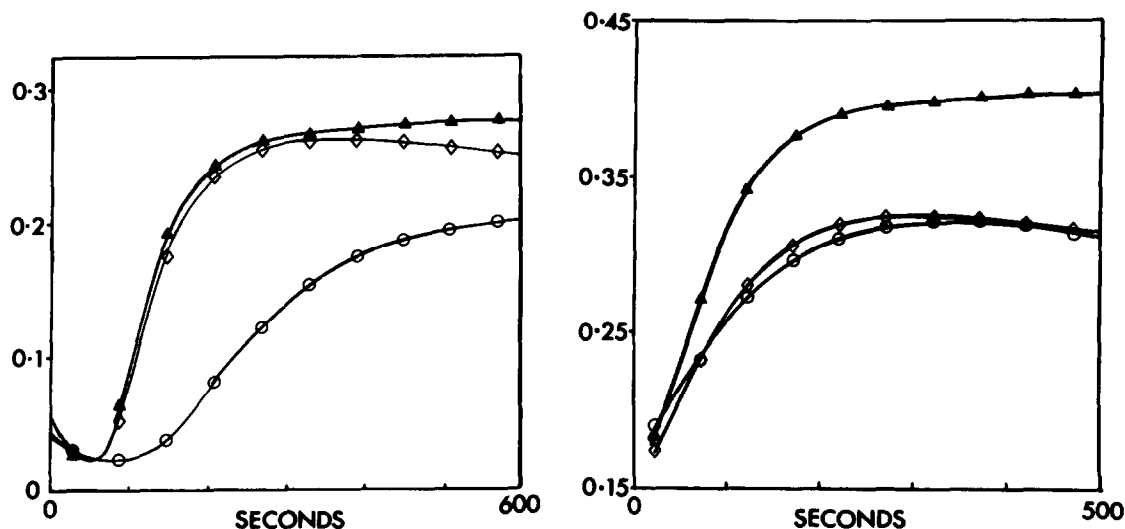


Fig.1. Effects of pyrophosphate ion and GTP on the assembly kinetics of microtubule protein, at 37°C in standard buffer. (a, left) Self-assembly of MT-protein, 1.2 mg/ml; (b, right) seeded assembly of MT protein, 0.9 mg/ml with 0.6 mg/ml microtubule seeds (see text). Symbols denote the presence of (○) 15 μM GTP; (◇) 15 μM GTP plus 0.5 mM pyrophosphate; and (▲) 0.5 mM GTP, as control.

assembled with $[GTP] = 15 \mu M$ (fig.2a) are substantially longer than the control (fig.2b) and the mean length has increased by 3-fold. The presence of 0.5 mM pyrophosphate (fig.2c) gives a length distribution closely similar to the control

with $[GTP] = 0.5 \text{ mM}$. Addition of 0.5 mM GTP to microtubule protein after attaining assembly plateau with $[GTP] = 15 \mu M$ showed additional assembly (1.4-fold) together with a 1.5-fold increase in mean length, consistent with the addi-

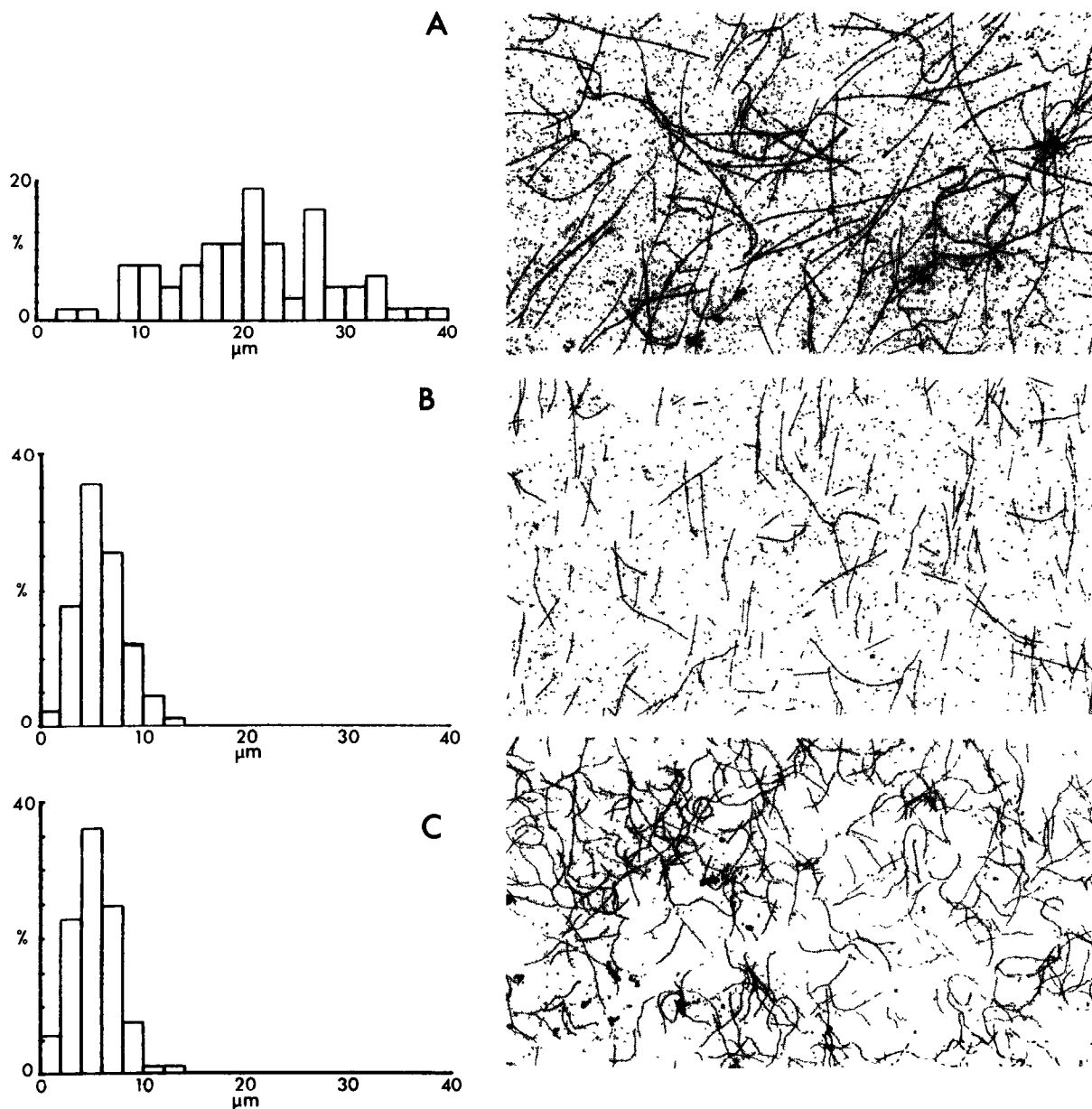


Fig.2. Electron micrographs and length distribution histograms of frequency vs length for microtubules formed under the conditions of fig.1a, with mean length (L) and number of measurements (N). (A) $[GTP] = 15 \mu M$; $L = 20.9 \mu m$, $N = 78$. (B) $[GTP] = 0.5 \text{ mM}$; $L = 5.9 \mu m$; $N = 89$. (C) $[GTP] = 15 \mu M$; $[pyrophosphate] = 0.5 \text{ mM}$; $L = 5.2 \mu m$; $N = 104$.

tional assembly occurring as elongation of existing microtubules.

Both the kinetic results and the marked reduction in mean microtubule length in the presence of pyrophosphate with $15\ \mu\text{M}$ GTP show that the principal effect of pyrophosphate is on the nucleation phase of microtubule assembly. Other compounds which enhance the rate of assembly with $[\text{GTP}] = 15\ \mu\text{M}$ and reduce the mean microtubule length significantly (i.e. >3 -fold) are tripolyphosphate, ATP, ADP or the non-hydrolysable analogue AMPPNP (all at $0.5\ \text{mM}$). By contrast, inclusion prior to assembly of GDP (up to $0.4\ \text{mM}$) together with GTP ($0.1\ \text{mM}$) inhibits the rate and amplitude of assembly, and enhances the mean length up to 4-fold.

4. DISCUSSION

The differential effects of GTP and GDP occupy a central role in control of microtubule assembly [1]. However, the requirement for GTP in assembly is not absolute; non-hydrolysable analogues of GTP are effective [1,6] and the assembly of nucleotide-depleted tubulin has recently been described [7]. In that study, and a related one with GMP-PCP induced assembly [6], we found a striking formation of short microtubules indicating efficient microtubule nucleation. Under both sets of conditions free GDP is effectively excluded. This, together with other evidence that, in the absence of free nucleotide, GDP could act sub-stoichiometrically to inhibit assembly [14] led us to investigate the role of GTP and GDP in the nucleation process.

When GTP is approximately stoichiometric with tubulin, reduced rate and amplitude of assembly are observed (cf. [15,16]). Our length determinations show that this is due to decreased nucleation. The exclusion of GDP, plus the effects of added nucleotides and anions clearly show that free GDP can reduce the spontaneous nucleation which occurs during GTP-induced self-assembly of microtubule protein. This inhibition can be relieved competitively by a number of polyphosphate containing compounds including nucleotide di- and triphosphates. Stimulation of assembly rates at low $[\text{GTP}]$ by added nucleotides evidently causes enhanced nucleation, and does not necessarily involve utilisation of the nucleotide at

the exchangeable nucleotide site.

The effect of GDP (added post-nucleation) on microtubule elongation and at steady state, mediated through nucleotide exchange reactions between tubulin-GTP and tubulin-GDP, does involve interaction of GDP with tubulin dimer at the exchangeable nucleotide-binding site [2–5]. The affinity for GDP is $K_a > 10^7$ [17]. Competition by pyrophosphate for E-site GDP indicates an affinity for this ion of $\sim 10^3$ [7]. The effects on nucleation shown by pyrophosphate suggest competition with a lower affinity binding of GDP. It is interesting that Zabrecky and Cole [18] have determined affinity constants for ADP and ATP of $\sim 10^4$ by direct binding [18]. Also the inhibitory effects at $[\text{GTP}] > 1\ \text{mM}$ suggest that additional weak binding sites may exist for nucleotide binding [2].

The inhibition by GDP of nucleation during self-assembly as demonstrated here explains several observed phenomena: (i) the effect of decreased $[\text{GTP}]$ on self-assembly of microtubule protein [15,16]; (ii) the pronounced inhibitory effects of GDP present with GTP prior to assembly [1,2,13,14,19]; (iii) the kinetic effects of added polyphosphates; (iv) the effects of nucleotides in enhancing the rate of GTP-induced assembly *in vitro*. In addition, GDP acts on microtubules in elongation phase and at steady state by producing a coupled equilibrium of GDP-tubulin with GTP-tubulin [2–5].

The effects reported here, excluding those in which GDP is explicitly added, generally involve only low concentrations of GDP, either displaced from the E-site of the G-25 protein or by hydrolysis of the (nearly) stoichiometric GTP used for assembly. Thus these effects relate almost exclusively to the influence of this GDP on the nucleation phase. A possible relevance to assembly processes *in vivo* of this inhibitory effect of GDP upon microtubule nucleation is that free GDP could act to prevent spontaneous formation of unwanted free microtubules. Such a control mechanism could serve to promote elongation of microtubules from endogenous cytoplasmic structures such as MTOCs, centrosomes or pre-existing microtubules.

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