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Assembly of microtubules with ATP: evidence that only a fraction of the protein is assembly-competent

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Chick brain microtubule protein can be assembled in vitro with ATP, although the extent of assembly is less than that with GTP. The ATP-induced assembly is not the result of generation of GTP by the co-purifying nucleoside diphosphate kinase. Neither an observed increase in the critical concentration nor the phosphorylation of MAP2 can account for the decreased extent of assembly. However, whereas microtubules are formed with both ATP and GTP, incubation with ATP yields additional filaments and polymorphic aggregates. The results demonstrate that of the total protein which can be assembled into microtubules by GTP, about 25–35% is assembled into other structural forms in the presence of ATP.

Microtubule *ATP-induced assembly* *Filament* *NDP kinase* *Microtubule protein* *GTP-induced assembly*

1. INTRODUCTION

The assembly of microtubules in vitro is generally considered to require GTP, which binds to an exchangeable site (E-site) on the tubulin dimer and which is hydrolysed on assembly with the resultant GDP becoming non-exchangeable [1–4]. However, non-hydrolysable GTP analogues such as GMPPNP and GMPPCP promote microtubule assembly following the partial removal of GDP, suggesting that they bind to the E-site and that GTP hydrolysis is not essential for assembly [1,5,6]. Furthermore, GDP also promotes assembly if nucleation sites are added, although the rate of elongation is much slower and the critical concentration is higher than with GTP [7].

Non-GTP nucleotide triphosphates such as ATP, CTP and UTP can also effect microtubule assembly even though they show no significant binding to the E-site [6,8,9]. This assembly has been postulated to be due to the formation of GTP by the co-purifying nucleoside diphosphate kinase (NDP kinase) utilising the non-GTP triphosphates as the phosphoryl donor and GDP originating from the E-site as the acceptor [1–3,8,9]. However, such a mechanism cannot account for the observed assembly, after the partial removal of GDP from the E-site, with non-hydrolysable ATP analogues such as AMPPNP and AMPPCP [10]. Similarly, we have shown that the observed assembly with CTP is not dependent upon the NDP kinase activity [11], suggesting that non-GTP nucleotide triphosphates may promote polymerisation by direct binding to the tubulin dimer. Indeed, it has been demonstrated [12] that ATP binds to the tubulin dimer ($k_d = 2 \times 10^{-4}$ M), and that this site is located on the α-tubulin subunit [13]. This site is therefore distinct from the GTP-binding site located on the β-subunit [14].

The effect of ATP on microtubule assembly is of particular importance as it is the dominant nucleotide triphosphate in vivo. In this paper, we examine the kinetics of microtubule assembly as a function of the ATP concentration and find that the observed assembly is not due to NDP kinase activity and that the extent of polymerisation with ATP is less than that with GTP. The results suggest that only a fraction of the microtubule protein which can be assembled with GTP is assembly-competent at physiological ATP concentrations.

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2. MATERIALS AND METHODS

2.1. *Preparation of microtubule protein*

Microtubule protein was purified from the brains of day-old chicks by cycles of assembly and disassembly as in [15]. The nucleotide content of the 3 × microtubule protein was reduced by fractionating on a Sephadex G-50 column (0.9 × 10 cm) pre-equilibrated in the assembly buffer. The protein eluting in the void fractions was used for the assembly studies, and had the same MAP2:tubulin composition as in [15,16].

The assembly buffer consisted of 0.1 M 4-morpholineethanesulphonic acid, 2.5 mM EGTA, 0.5 mM $MgSO_4$, 0.1 mM EDTA, 1.0 mM dithiothreitol, pH 6.4 with KOH.

2.2. *Microtubule assembly*

Assembly was monitored at 37°C by measuring the change in absorption at A_{350nm} using a Beckman DU-8 spectrophotometer fitted with a temperature-controlled cuvette holder [15,17]. The change in absorption is directly proportional to the extent of microtubule assembly as measured by the amount of pelletable protein [17].

2.3. *Electron microscopy*

Protein samples were negatively stained with 1% uranyl acetate, using Formvar/carbon coated grids, and viewed with a Philips 300 electron microscope at 80 kV.

2.4. *Other methods*

Protein concentrations were determined by the method of [18] using bovine serum albumin as the calibration standard. The nucleotide content of the microtubule protein was determined as described in [15], while the NDP kinase activity was determined using the couple enzyme procedure according to [19].

2.5. *Materials*

Biochemicals were purchased from Sigma and Sephadex G-50 from Pharmacia. All other reagents were of Analar grade.

3. RESULTS

The elution of 3 × microtubule protein through a Sephadex G-50 column typically reduces the GDP content to about 0.4 mol GDP·mol^{-1} tubulin dimer, representing the complete removal of GDP bound to the tubulin dimer E-site, while the GDP bound to the slowly exchangeable site on MAP2:tubulin oligomers, which constitute about 55% of the total protein [15], is mainly retained. This protein has been used to examine the effects of GTP and ATP on the kinetics of microtubule assembly.

The assembly was examined by incubating microtubule protein (0.87 mg·ml^{-1}) at 37°C in the presence of 50 μM GTP or 50 μM ATP. The assembly with ATP exhibited a longer lag period than with GTP and the maximum rate of elongation was considerably slower. In addition, the maximum extent of assembly was significantly lower with ATP than that with GTP (fig.1). The addition of 50 μM GTP to the protein assembled with ATP only slightly increased the extent of polymerisation, and this was followed by a steady decrease in the extent. By contrast, the addition of 50 μM ATP to the protein assembled with GTP induced an immediate depolymerisation similar to that reported in [17]. A control sample which had

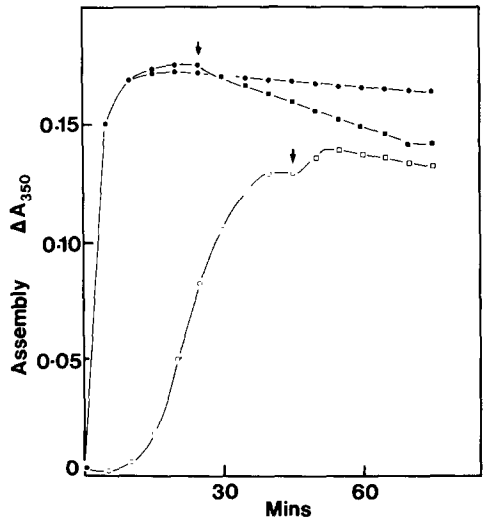

Fig.1. Kinetics of microtubule assembly in the presence of ATP and GTP. 3 × microtubule protein (0.87 mg·ml^{-1}) was incubated at 37°C in the presence of either 50 μM GTP (●) or 50 μM ATP (○) monitoring the assembly kinetics by absorption at A_{350nm}. When the assembly had reached the maximum extent, 50 μM ATP was added to the GTP-sample (■) or 50 μM GTP to the ATP-sample (□), as arrowed.

been preincubated for 40 min without added nucleotide assembled to the full extent on addition of 50 μM GTP. The decrease in the extent of assembly with ATP and its non-reversal with GTP cannot therefore be attributed to a time-dependent inactivation of the protein, and this can also be concluded from the observed decrease following the addition of ATP to the GTP sample.

The decrease observed with ATP could be due to a higher critical concentration [20]. Increasing concentrations of microtubule protein were assembled with either 50 μM ATP or 50 μM GTP, and the maximum extent of assembly determined. The ATP-induced polymerisation does indeed exhibit a higher critical concentration than with GTP (0.17 mg·ml^{-1} *vs* 0.085 mg·ml^{-1}, fig.2). A difference in the critical concentration should yield, in measurements of the maximum extent, a series of parallel lines: the extents with GTP and ATP diverge with increasing protein concentrations (fig.2), indicating that a fraction of the protein cannot be assembled with ATP. In particular, the difference in the critical concentration is equivalent to a decrease in the maximum extent of assembly at 0.92 mg·ml^{-1} of only 10.2%, less than the observed 27% decrease for this protein preparation (fig.1), but equal to the increase observed on addition of GTP to the ATP-sample.

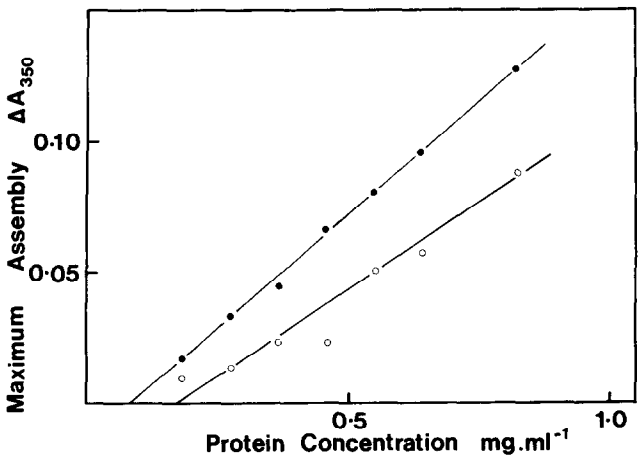

Fig.2. Critical concentration for microtubule assembly with ATP and GTP. Increasing concentrations of 3 × microtubule protein was incubated at 37°C with 50 μM ATP (○) or 50 μM GTP (●), and the maximum extent of assembly determined by absorption at A_{350nm}.

Decreases of 25–35% have been observed using different preparations of microtubule protein.

The change in the critical concentration is unlikely to be due to the phosphorylation of MAP2, which does increase the critical concentration [17], as phosphorylation is highly dependent upon the phosphoryl donor concentration and is minimal at 50 μM [21]. In addition, the increase in the critical concentration is the same when determined using the initial rates of assembly (not shown) when the extent of phosphorylation is minimal.

We have therefore examined the final extent of assembly as a function of the GTP and ATP concentrations, maintaining the protein concentration at 0.92 mg·ml^{-1}. The extent of assembly increases as the GTP concentration is raised (fig.3), attaining a maximum value at 100 μM GTP. The extent declines at higher GTP concentrations, and this may indeed be due to MAP2 phosphorylation as GTP is also a donor for the protein kinase [22].

The maximum extent of assembly as a function of the ATP concentration is significantly different to that observed with GTP (fig.3). The extent increases to a value at 10 μM ATP close to that observed with GTP, while higher ATP concentrations (20–500 μM) result in a 32% decrease in the extent of assembly. Higher ATP concentrations (above 500 μM) induce a further reduction, as observed with GTP, and which again is probably due to MAP2 phosphorylation. There is therefore

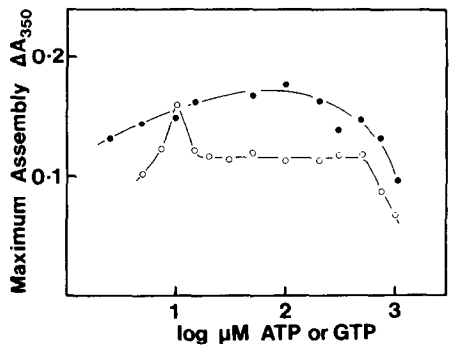

Fig.3. Maximum extent of microtubule assembly as a function of the ATP and GTP concentrations. 3 × microtubule protein (0.92 mg·ml^{-1}) was incubated at 37°C with increasing concentrations of ATP (○) or GTP (●) and the maximum extent of assembly determined by absorption at A_{350nm}.

a significant inhibition in the extent of microtubule assembly by 20–500 μM ATP compared with the extent with GTP, an inhibition which cannot be wholly attributed to an increase in the critical concentration for assembly (fig.2).

If assembly by non-GTP nucleotide triphosphates is mediated by NDP kinase charging up the tubulin E-site GDP, the observed lower extent of assembly with ATP might be due to the substoichiometric GDP concentration resulting from the gel filtration pretreatment. However, the GDP concentration is decreased by 50–60%, i.e., more than the observed ATP effect (figs 1 and 2). Comparison of the assembly of microtubule protein (0.82 mg·ml^{-1}) at 50 μM ATP and 5 μM GTP shows that the rate of polymerisation is similar although the ATP-sample has a longer lag phase (fig.4). Furthermore, the lag phase is also longer with 50 μM ATP than with 1 μM GTP (4 min *vs* 3 min, fig.4 inset), but the initial rate of elongation is now faster with ATP (fig.4). If GTP-generation

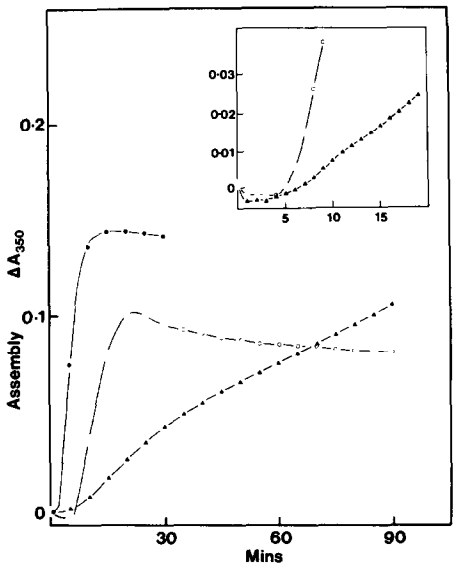

Fig.4. Kinetics of microtubule assembly with 50 μM ATP or 1 μM GTP. 3× microtubule protein (0.82 mg·ml^{-1}) was incubated at 37°C in the presence of either 50 μM ATP (○), 5 μM GTP (●) or 1 μM GTP (▲), and the assembly kinetics monitored by absorption at A_{350nm}. Inset: an expansion of the initial time period showing the shorter lag period with 1 μM GTP than with 50 μM ATP, while the initial rate of assembly was greater with ATP than with GTP.

limits the assembly by ATP then both the duration of the lag phase and the initial rate of elongation should be equally affected, although the subsequent rate of elongation could be differentially affected as the extent of GTP conversion would increase with time.

The NDP kinase activity of 3× microtubule protein is only 15–20 nmol GTP generated·min^{-1}·mg^{-1} protein, assayed under non-limiting conditions. However, NDP kinase functions via a 'ping pong' mechanism [23,24] with the reaction rate depending upon both the donor and acceptor concentrations. In the above experiment the ATP and GDP concentrations were 50 μM and 3 μM respectively, considerably lower than the k_m^{ATP} of 0.5 mM [24], so that the rate of GTP generation would be severely restricted. Preliminary calculations on the basis of the observed chick brain NDP kinase k_m^{ATP} and k_m^{TDP}, the respective values of V_{max}, and the efficiency of utilisation of GTP compared with ATP, indicate that less than 0.6 μM GTP could have been generated within a 10 min incubation period. Furthermore, the reproducibly observed higher level of assembly at 10 μM ATP than at higher ATP concentrations (fig.2) is inconsistent with a dependency on NDP kinase generation of GTP. Similarly, if NDP kinase activity were required for the ATP-induced assembly then the reduction in the extent of assembly, compared with GTP, should be reversed by the addition of 50 μM GTP. This is not observed (fig.1).

The results therefore demonstrate that ATP can directly induce some microtubule assembly. Indeed, non-hydrolysable ATP analogues can also promote assembly [10] yet clearly they cannot be substrates for the NDP kinase. Finally, we have demonstrated that CTP can induce assembly, and that the lag period is short even though CTP is a very poor substrate for NDP kinase [11].

As the maximal extent of ATP-induced assembly, determined by light scattering, is significantly lower than that with the equivalent GTP concentration, only a fraction of the total protein is apparently assembled by ATP into microtubules. This has been examined by electron microscopy of negatively stained samples assembled to equilibrium with 50 μM ATP or 50 μM GTP (fig.5). Microtubules of normal morphology were observed with both ATP and GTP. The GTP-

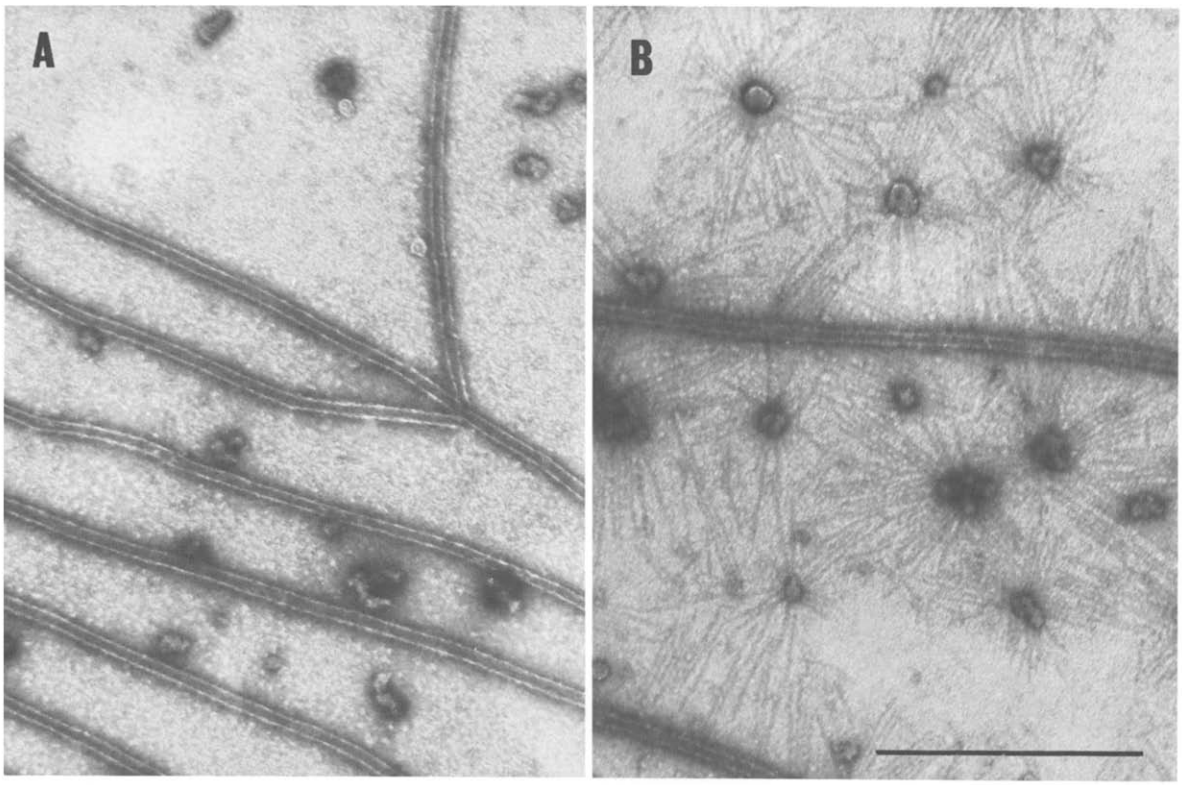

Fig.5. Electron microscopy of the GTP- and ATP-assembly products. 3 × microtubule protein (0.9 mg·ml^{-1}) was incubated at 37°C with either (A) 50 μM GTP or (B) 50 μM ATP and assembled until equilibrium. Samples were fixed with 0.9% glutaraldehyde, diluted 10-fold, and negatively stained. The assembly with GTP shows intact microtubules, globular particles, and a uniform background of unpolymerised protein. The ATP-sample contains additional short filaments radiating out from the globular particles. Bar = 0.5 μm.

sample showed a fairly uniform background of unpolymerised protein, and unidentified globular particles (fig.5A). By contrast, the ATP-sample consistently contained discrete filaments, which frequently aggregate to form 'aster-like' assemblies radiating out from the globular particles (fig.5B). The addition of ATP therefore results in the formation of both microtubules and these assembly-incompetent polymorphic aggregates while microtubules are the principle assembly product with GTP.

4. DISCUSSION

ATP induces the assembly of microtubules, and this is not due to the generation of GTP by co-purifying NDP kinase charging up the GDP bound to the tubulin E-site. There have been previous reports that ATP can induce microtubule assembly (e.g., [9,25,26]), but this is the first demonstration of a direct effect of ATP. It is though consistent with the earlier observation that non-hydrolysable ATP analogues can promote polymerisation [10].

However, the extent of microtubule assembly is significantly (25–35%) less than that observed with GTP (figs 1–3), and this cannot be accounted for by either the higher critical concentration for ATP-induced assembly (fig.2) or by the phosphorylation of MAP2. Instead, the results suggest that only a fraction of the total protein is competent, compared with GTP, of being assembled by ATP. Furthermore, the addition of 50 μM GTP to

microtubules pre-assembled with ATP fails to restore the level of assembly to that observed with 50 μM GTP (fig.1). By contrast, the addition of 50 μM ATP to microtubules pre-assembled with GTP induced an immediate depolymerisation (fig.1), at a rate which is governed by the rate of MAP2 phosphorylation [17].

Electron microscopy shows that the samples assembled with ATP contain short filaments and polymorphic aggregates which are not observed after assembly with GTP (fig.5). Such aggregates would not scatter light significantly and therefore would only marginally affect the turbidimetric measurement of microtubule assembly [25]. The observed decrease in the extent of assembly with ATP can therefore be attributed to the formation of these polymorphic structures. They differ from the rings formed on incubating pure tubulin with ATP [27], and this may be due either to the presence of MAP2 or to the lower magnesium concentrations used in the current study. Significantly, the addition of GTP to these ATP-induced aggregates resulted, as in the present study, in a mixture of intact microtubules and unaltered aggregates [27].

The fraction of protein which cannot be assembled with ATP may represent specific tubulin isoforms or post-translational modifications. It is therefore interesting to note that the α-tubulin subunit is the substrate for tubulin-tyrosine ligase [28], that this subunit also binds ATP [12,13], and that tyrosylation requires ATP hydrolysis [29]. One possibility therefore is that the protein not assembled into microtubules by ATP is the detyrosylated fraction.

Finally, both the assembly and inhibition by ATP is observed over a wide concentration range (fig.3). This suggests that both GTP and ATP may be involved in the in vivo regulation of microtubule assembly, particularly as the cellular ATP concentration is 4–7-fold higher than that of GTP [30,31].

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