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MECHANISM OF GTP HYDROLYSIS AT MICROTUBULE ENDS

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The kinetics of tubulin subunits incorporation into microtubules and the kinetics of inorganic phosphate release have been measured in parallel. Correlation of the two measurements indicates that the tubulin GTPase activity is due to GTP hydrolysis and exchange at the end of the microtubules. In some cases where the free GTP available in the medium is insufficient the rate of GTP hydrolysis is limited by the rate of tubulin-GTP association at the end of the microtubules. The affinity constant of GTP for the microtubule end appears to be 100 times lower than the affinity constant of the tubulin-GTP complex.

1. Introduction

The building subunit of microtubules is a tubulin dimer which possesses two binding sites for the guanine nucleotides [1]. On one site, the "N" site, one finds almost exclusively GTP which exchanges very slowly if not at all [2] and which is not hydrolysed during polymerisation [3,4]. On the other site, the "E" site, GTP as well as GDP can bind reversibly. Dissociation constants for GTP and GDP varying between 10^{-6} to 10^{-8} mol ℓ^{-1} have been published in the litterature [5–7]. The "E" site also appears to be specific for the guanine nucleotides since it does not bind significantly ATP, UTP nor CTP [2,8]. The binding of GTP or of a non hydrolysable analog of GTP to the "E" site is required to initiate microtubule assembly [2–4,9–11]. However in the presence of GDP and ATP, UTP or CTP, microtubules will eventually form. This is due to the presence in the tubulin preparations of a NDP kinase activity [2,4,12] which catalyzes the phosphorylation of GDP or T-GDP into GTP or T-GTP according to the following reaction:

$$\begin{matrix}\text{GDP}\\ \text{or}\\ \text{T-GDP}\end{matrix} + \text{ATP*} \xrightleftharpoons{\text{NDP-kinase}} \begin{matrix}\text{GTP*}\\ \text{or}\\ \text{T-GTP*}\end{matrix} + \text{ADP.}$$

The isolated tubulin dimer is devoid of GTPase activity. But during the course of assembly of pure tubulin into microtubules, a GTPase activity is observed which can be attributed to tubulin itself and which depends on the number of microtubule ends [13,14]. It is also known that the previously exchangeable "E" site of the tubulin subunit has become nonexchangeable and contains exclusively GDP inside the microtubule [3].

From these last informations, it was suggested that GTP hydrolysis at the "E" site of tubulin was occurring as a tubulin subunit was polymerising onto the end of a microtubule [13]. The possibility that GTP exchange and hydrolysis could occur on the "E" sites at the end of the microtubules could not however be rejected. The exchangeability of GTP at the microtubule end "E" sites was not evident to demonstrate since these sites on a 4 μm length microtubule represent only one over 300 of the total microtubule "E" sites.

The question which remained was then: is the tubulin GTPase activity due to the tubulin-GTP association at the end of the microtubules or is it due to GTP exchange and hydrolysis at the same ends?

If one considers the problem from the view point of the tubulin GTPase activity, the two models can be differentiated theoretically. In the first case, the GTPase activity depends on the tubulin-GTP concentration but not on the free GTP concentration. In the

second case, on the contrary, it depends on the concentration of free GTP in solution relative to the dissociation constant of the complex microtubule end-GTP.

In practice, to solve the problem, it was necessary to find a stable polymerising system in which one could vary the free GTP concentration without varying the tubulin-GTP concentration. In order to obtain stable microtubules with very low free GTP in solution, it was necessary to work with a regenerating system. Indeed, the microtubules are not stable at low GTP concentrations [15] due to the GTP hydrolysis which accompanies their formation. We chose then to use the regenerating system [ATP + GDP + NDP kinase].

Owing to this study, it has been possible to demonstrate that the tubulin GTPase activity is due to GTP hydrolysis and exchange on the "E" site of the tubulin subunits located at the end of the microtubules.

2. Materials and methods

2.1. Materials

The radioactive compounds, [^{3}H]GTP and [γ^{32}P]-GTP were purchased from Amersham-Searle Corporation. EGTA (ethylene-bis-(β aminoethyl-ether) N,N'-tetraacetic acid) was a Sigma product, MES (2-(N-morpholino)ethane sulfonic acid) was from Calbiochem and unlabeled GTP from Boehringer-Mannheim.

2.2. Preparation of microtubule protein

Microtubule protein was isolated from pig brain homogenates by two cycles of polymerisation and depolymerisation as described by Shelanski et al. [16] and stored in RB buffer (0.1 M MES, 0.5 mM $MgCl_2$, 0.5 mM GTP, 1 mM EGTA, pH 6.6) containing 4 M-glycerol at −20°C. Before experimentation a third assembly cycle was performed. Microtubules were pelleted at 25°C for 30 min at 120 000 g and resuspended in glycerol-free RB buffer for 30 min at 4°C to obtain depolymerisation. The insoluble aggregates were removed by centrifugation for 15 min at 20 000 g, 4°C.

Further purification of tubulin and isolation of the microtubule associated proteins was carried out on a phosphocellulose column according to the procedure of Weingarten et al. [17]. Prior to use, Whatman phosphocellulose P11 was washed successively with 0.5 M NaOH, H_2O, 0.5 M HCl, H_2O and equilibrated with RB buffer diluted twice (RB/2). No more than 2 mg protein per ml of phosphocellulose were applied and the flow rate was of about one column volume per hour. Phosphocellulose purified tubulin is devoid of most of the microtubule associated proteins [18] but it still contains a NDP kinase activity [18,19]. In order to obtain the polymerisation of the phosphocellulose purified tubulin by the system NDP-kinase-ATP-GDP, the free GTP remaining in the preparation was first hydrolysed through several cycles of polymerisation-depolymerisation in a buffer at pH 6.6 containing 0.05 M MES, 0.5 mM EGTA, 10 mM Mg^{2+} and 30% glycerol. Then, the tubulin preparation was passed through a Sephadex G25 column in order to eliminate the excess GDP. After chromatography, the tubulin preparation contained 2 moles of guanine nucleotide per mole of tubulin. By thin layer chromatography, it has been shown that, under these preparation conditions, the tubulin binds one GTP on its "N", site and one GDP on its "E" site [18].

2.3. Inorganic phosphate liberation

The liberation of ^{32}P labeled inorganic phosphate was measured according to the procedure of Nishizuka et al. [20]. Before experimentation, free nucleotides were removed by adsorption on activated charcoal or by passage through on a column of Sephadex G25 and the samples were preincubated at 4°C in the presence of a given concentration of [γ^{32}P]-labeled GTP for at least 20 min. The release of inorganic phosphate was found not to occur during the period of preincubation at 4°C. Like the polymerisation reaction, the reaction of GTP hydrolysis was started by a temperature jump from 4°C to the desired temperature.

In order to obtain kinetics of GTP hydrolysis, 50 μl aliquots of the sample were removed at different times and put into 450 μl perchloric acid solution (0.25 N) to quench the reaction. Then 1 ml of 600 μM KH_2PO_4 was added as carrier followed by the addition of 500 $\mu\ell$ of 5% ammonium molybdate in 4 N H_2SO_4. The phosphomolybdate complex formed was extracted into a 2 ml isobutanol-cyclohexane mixture (1 : 1 v/v). 1 ml of the organic phase was removed, mixed with a 10 ml Bray solution and counted in a

Packard counter. Protein concentration was determined by the method of Lowry et al. [21] with a correction of 10% for the coulour difference between tubulin and BSA [13]. The rate of GTP hydrolysis during tubulin self assembly is maximum at the beginning of the polymerisation reaction. Then, it slows down until it reaches a steady state at about the same time or a little after the turbidity plateau had been reached [13]. In this paper, the term "burst" will refer to the early and rapid inorganic phosphate release characterizing the kinetics of GTP hydrolysis.

The phosphocellulose purified tubulin used in the experiments described here was shown to be devoid of GTPase activity under non-polymerizing conditions i.e. when the polymerisation reaction was inhibited by an excess of vinblastine. Notice that it has been shown earlier that vinblastine does not inhibit the GTPase activity of the microtubule associated proteins under concentration conditions where it inhibits the tubulin polymerisation and GTPase activity [31].

2.4. Polymerisation kinetics

The polymerisation of tubulin *in vitro* was monitored by turbidimetry at 350 nm on a Beckman Acta V spectrophotometer in cells of 0.5 cm lightpath. The polymerisation reaction was started by a temperature jump from 4°C to 37°C which required an half time of 4 s. Temperatures were controlled to within 0.1°C by a thermostatically regulated liquid circulator. The shape of the polymerisation curves obtained by turbidimetry have been all characterized [22]: there is a brief lag time which precedes a rapid increase in turbidity and eventually turbidity reaches a plateau as an apparent equilibrium is attained.

According to Berne [22] the light scattering intensity (Δ) of a solution of polydisperse, long and thin rods like the microtubules should be proportional to the concentration of tubulin incorporated into the microtubules, (C_w): $\Delta = const.\ C_w$, *provided:* a) the length (L) of the microtubules be larger than the wavelength of the incident light (λ) and b) the thickness (t) of the microtubule be small compared to λ.

It has been shown that the approximation holds quite well at 350 nm wavelength for a solution of microtubules of 250 nm thickness and 4 μm average length and even for microtubules less than 0.8 μm length [22].

We shall therefore assume in this report that the conditions a) and b) are satisfied all over the turbidity curves.

3. Theory. Mechanisms of GTP hydrolysis at the microtubule ends

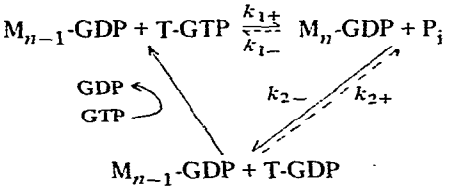
The experimental observations that 1) the GTPase activity of the microtubule solution at equilibrium is proportional to the number of microtubule ends [13], 2) the isolated tubulin subunits which coexist at equilibrium with the microtubules do not possess a GTPase activity [14], can be accounted for by two models:

1) GTP hydrolysis occurs each time a tubulin subunit carrying a GTP on its "E" site polymerises onto the end of a microtubule.

2) GTP hydrolysis occurs each time a GTP binds to an "E" site at the end of a microtubule.

3.1. First model

GTP hydrolysis occurs each time a tubulin subunit carrying a GTP on its "E" site polymerises onto the end of a microtubule. This model can be illustrated in the following way:



T-GTP and T-GDP stand for the GTP- and GDP-tubulin complexes and M_{n-1} or M_n represent microtubules with a degree of polymerisation $n-1$ or n, respectively.

In this model we shall consider the reactions involving k_{1-} and k_{2+} as negligible. Neglecting k_{1-} is justified owing to the observation that the dissociation of a tubulin subunit from the microtubule ends is not accompanied by a phosphorylation of GDP to form GTP [13]. Concerning the non-polymerisability of the T-GDP complex into microtubules, contradictory results have been reported [15,23,24]. Anyway, the affinity constant of the T-GDP complex is 3 to 5 times lower than the affinity constant of the T-GTP

complex [7,11]. In addition, we shall be always working at GTP concentrations large compared to the GDP concentration so that the T-GDP concentration will always be small compared to the T-GTP concentration. Furthermore, the authors claiming that the tubulin-GDP complex would be able to elongate microtubules [15,23], also reported that the elongation rate of tubulin-GDP would be not more [15] if not less [23] than the elongation rate of the tubulin-GTP complex. All these remarks justify that k_{2+} be neglected. Notice that the model does not consider the possibility that a T-GTP complex could dissociate from the microtubule end before the GTP on its "E" site had been hydrolysed. This assumption is justified by the experiments since we never found an initial rate of GTP hydrolysis during polymerisation which would have been lower than the corresponding rate of T-GTP association.

The kinetics of polymerisation can be treated by a method similar to that developed in the field of polymer chemistry [25]: the rate of growth of a p-mer to an $(p+1)$-mer by attachment of a free monomer is expressed as $k_{1+}c_p(t)c_1(t)$; the rate of depolymerisation of the p-mer into a $(p-1)$mer by removal of a monomer from the polymer end is expressed as $k_{2-}c_p(t)$ [26,27].

If one neglects k_{-1} and k_{2+}, the rate of incorporation of tubulin subunits into microtubules is equal to:

$$dc_1(t)/dt = -k_{1+}c_p(t)c_1(t) + k_{2-}c_p(t)$$

$$= k_{1+}c_p(t)[\bar{c}_1 - c_1(t)], \quad (1)$$

where c_1 is the free tubulin-GTP concentration and c_p, the microtubule concentration. (t) indicates that both c_1 and c_p are dependent on time.

$\bar{c}_1 = k_{2-}/k_{1+}$ is the critical tubulin subunit concentration, that is, the tubulin subunit concentration which is present with microtubules at steady state, when the rates of tubulin dimer association and dissociation are equal. In the case where c_p does not vary with time, the solution of eq. (1) is:

$$c_1(t) = \bar{c}_1 + (c_{tot} - \bar{c}_1)e^{-k_{1+}c_p t} \quad (2)$$

where c_{tot} is the total tubulin concentration.

Within the first model, the rate of inorganic phosphate release is determined by the frequency of tubulin subunit association to the microtubule end:

$$dP_i(t)/dt = k_{1+}c_p(t)c_1(t). \quad (3)$$

From eq. (1), we can extract:

$$k_{1+}c_p(t) = \frac{dc_1(t)/dt}{\bar{c} - c_1(t)}. \quad (4)$$

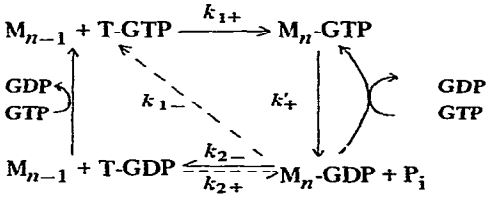
Substituting eq. (4) into (3) leads to:

$$\frac{dP_i(t)}{dt} = \frac{dc_1(t)}{dt}\,\frac{c_1(t)}{[\bar{c}_1 - c_1(t)]}. \quad (5)$$

Eq. (5) correlates the kinetics of tubulin subunit concentration with the kinetics of inorganic phosphate release.

3.2. Second model

GTP hydrolysis occurs each time a GTP binds to an "E" site at the end of a microtubule according to the following scheme:



For the same grounds as in the first model, we make the assumption here that T-GTP dissociation from the microtubule ends cannot occur and we neglect k_{2+} and k_{1-}.

The rate of incorporation of tubulin dimers into microtubules is the same as in the first model but the rate of inorganic phosphate release is modified and becomes:

$$dP_i(t)/dt = k'_+[M_n - GTP](t). \quad (6)$$

Several cases are possible:

a) The GTP exchange and hydrolysis at the microtubule ends is faster than the tubulin-GTP turnover and the microtubule ends are saturated with GTP – that is, if the following conditions are satisfied:

$$GTP \gg K_{d,M_n,GTP} \quad (7)$$

where $K_{d,M_n,GTP}$ is the dissociation constant of the M_n-GTP complex into M_n and GTP.

$$[M_n\text{-GDP}] \neq 0 \quad (8)$$

then

$$dP_i(t)/dt = k'_+ c_p(t) = k_{1+} c_p(t) k'_+ / k_{1+} \quad (9)$$

b) The concentration of free GTP in solution is negligible compared to $K_{d,M_n,GTP}$. But still, the T-GTP concentration is kept constant (owing to a regenerating system for instance) so that the polymerisation equilibrium can be maintained. In this case, the concentration of $[M_n\text{-GTP}]$ is determined by:

$$d[M_n\text{-GTP}](t)/dt$$

$$= k_{1+} c_p(t) c_1(t) - k'_+ [M_n\text{-GTP}](t). \quad (10)$$

It can be shown that, even in the case where $d[M_n\text{-GTP}](t)/dt \neq 0$, the solution of the eq. (10) approximates to (see appendix):

$$[M_n\text{-GTP}](t) = (k_{1+}/k'_+) c_p(t) c_1(t). \quad (11)$$

Substituting eq. (11) into (6) leads to eq. (3):

$$dP_i(t)/dt = k_{1+} c_p(t) \tilde{c}_1(t).$$

One sees that this situation comes back to that of the first model. In the second model however the situation depicted corresponds to a limiting case which arises at free GTP concentrations low compared to the dissociation constant of the M_n-GTP complex. On the contrary if the first model applies, eq. (3) should be valid at any GTP concentration provided the T-GTP concentration be kept unchanged.

c) In general,

$$[M_n\text{-GTP}] = c_p(t) - [M_n] - [M_n\text{-GDP}] \quad (13)$$

that is, the rate of inorganic phosphate release will be a complex function of the dissociation constant of the complexes M_n-GTP and M_n-GDP and of the concentrations of GTP and GDP. We shall not deal with the general case here.

4. Experimental results

4.1. Effect of free GTP on the steady state rate of GTP hydrolysis

Phosphocellulose purified tubulin (17.5 μM) containing one GTP on its "N" site and one GDP on its "E" site (see sect. 2) did not polymerise at 37°C in a RB/2 buffer with 10 mM Mg^{2+} and 30% glycerol (RBMG buffer). Under the same buffer conditions, after preincubation at 4°C with 500 μM ATP during two hours, the polymerisation reaction was induced by a temperature jump to 37°C and a turbidity curve analogous to those presented in figs. 2A and 3A was obtained. Under the electron microscope, a mixture of microtubules and sheets could be observed. In a second sample polymerising under the same conditions as just described was added 300 μM GTP after a 30 min incubation at 37°C, that is after the turbidity plateau had been reached. No change in the turbidity level could be detected. On the other hand, the amount of polymerised material, as measured after centrifugation of the samples during 20 min at 160 000 g and 37°C, was exactly the same before and after addition of the 300 μM GTP. In the present case, the tubulin concentration was 8.5 μM in the pellet and 9 μM, in the supernatant. These observations indicated that adding 300 μM GTP in the sample did not change the amount of tubulin-GTP coexisting at equilibrium with the tubulin polymers. In other words, the quantity of NDP-kinase and ATP present in the sample polymerising in the absence of GTP added was sufficient to phosphorylate all the remaining GDP (one equivalent per tubulin dimer) into GTP. Moreover the quantity of GTP obtained through the transphosphorylation reaction was sufficient to saturate the tubulin subunits and obtain the maximal level of polymerisation. This last conclusion is not wondering if one considers the high affinity of GTP for the tubulin exchangeable site. In consequence, the concentration of free GTP remaining in solution was low.

For the GTP hydrolysis experiments, the same tubulin preparation as previously was separated into two batches. One sample was preincubated in the presence of 500 μM [γ-^{32}P]-labeled ATP, the other in the presence of 500 μM unlabeled ATP. The two samples were induced to polymerise at the same time and the time course of inorganic phosphate release was followed from time 0 to 80 min on the first sample. In the second sample, 300 μM [γ-^{32}P]-labeled GTP was added after 30 min at 37°C and the time course of inorganic phosphate release was followed from that time to 80 min. The result of the experiment is plotted on fig. 1. One can see that the steady

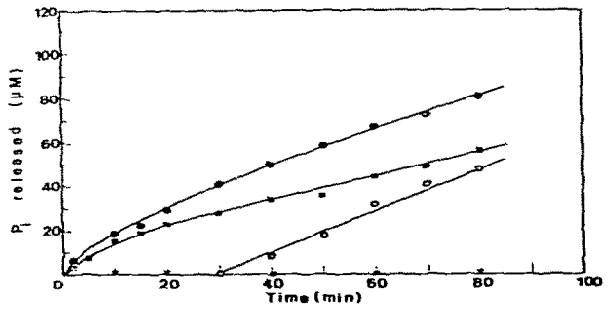


Fig. 1. Time course of inorganic phosphate release during the polymerisation of 17.5 μM phosphocellulose purified tubulin in RBMG buffer at 37°C, in the preesnce of 300 μM [γ-^{32}P] labeled GTP (•); in the presence of 500 μM [γ-^{32}P] labeled ATP (▪); in the presence of 500 μM unlabeled ATP and 300 μM [γ-^{32}P] labeled GTP added 30 min after the induction of the polymerisation reaction (○). Control experiment (X) showing the absence of GTPase activity in the absence of polymerisation, i.e. when 20 μM vinblastine were added to the 17.5 μM phosphocellulose purified tubulin in RBMG buffer at 37°C in the presence of 300 μM [γ-^{32}P] labeled GTP or 500 μM [γ-^{32}P] labeled ATP

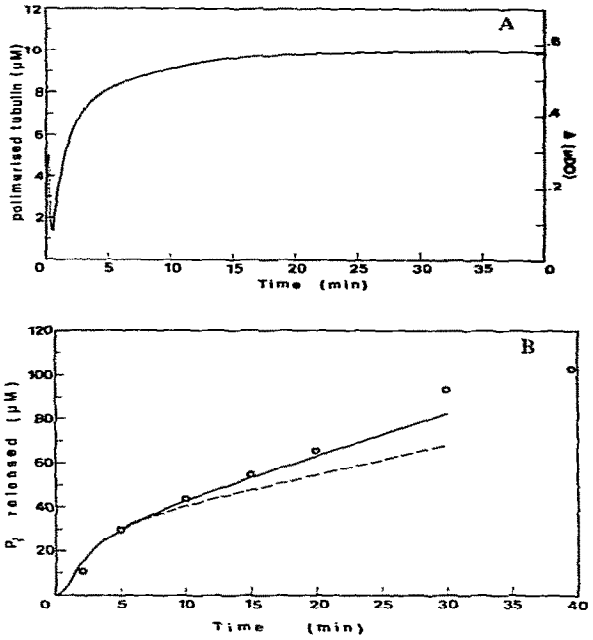


Fig. 2. (A). Kinetics of polymerisation at 37°C of 22 μM phosphocellulose purified tubulin in RBMG buffer and in the presence of 300 μM GTP. (B). Kinetics of GTP hydrolysis during the course of polymerisation of tubulin under the experimental conditions described in (A). Experimental points (○). Theoretical curves derived from the polymerisation kinetics shown in (A) after eq. (3) (– – –) and after eq. (9) (——).

state rate of GTP hydrolysis is different in the absence and in the presence of excess free GTP added (0.54 and 0.96 μM min^{-1} respectively). Thus the steady state rate of GTP hydrolysis depends on the concentration of free GTP in solution. This experiment favours then the second model "GTP hydrolysis and exchange at the end of the microtubules". The GTP hydrolysis kinetics obtained in the presence of [γ-^{32}P]-labeled ATP and a stoichiometric amount of GDP to tubulin correspond to the case b) where the free GTP concentration is low compared to the dissociation constant of the complex M_n-GTP. After the addition of 300 μM [γ-^{32}P], it corresponds to the case a) where the free GTP concentration is large compared to $K_{d,M_n,GTP}$. Therefore, the steady state rate of GTP hydrolysis in the presence of ATP and in the presence of ATP plus GTP can be derived from eqs. (3) and (9) respectively:

$$dP_i/dt = k_{1+}c_p\bar{c}_1 = 0.54\ \mu M\ min^{-1}$$

and

$$dP_i/dt = k_{1+}c_p k'_+/k_{1+} = 0.96\ \mu M\ min^{-1}.$$

It is reasonable to think that the number of microtubules has not drastically changed after the 300 μM GTP addition. This was confirmed by observation under the electron microscope – therefore:

$$\frac{k'_+}{k_{1+}} = \frac{0.96}{0.54}\ \bar{c}_1 = 16\ \mu M, \quad \text{since } \bar{c}_1 = 9\ \mu M.$$

The polymerisation and GTP hydrolysis kinetics of the 17.5 μM tubulin preparation was also studied in the presence of 300 μM [γ-^{32}P]-labeled GTP alone. The polymerisation rate was maximal under these conditions. Also here, the same turbidity plateau was reached and the amount of polymerised tubulin was equal to 8.5 μM. The kinetic of GTP hydrolysis under these conditions is shown in fig. 1. The quantity of inorganic phosphate release at a given time is always higher during polymerisation in the presence of 300 μM GTP than in the presence of 500 μM ATP. Moreover the steady state rate of GTP hydrolysis (0.88 μM min^{-1}) is nearly equal to the steady

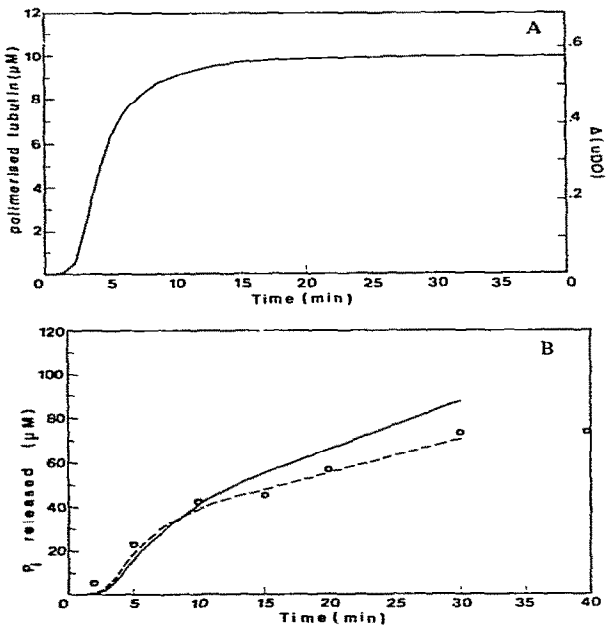


Fig. 3. (A). Kinetics of polymerisation at 37°C of 22 μM phosphocellulose purified tubulin in RBMG buffer and in the presence of 500 μM ATP and a stoichiometric amount of GDP to tubulin. (B). Kinetics of GTP hydrolysis during the course of polymerisation of tubulin under the experimental conditions described in (A). Experimental points (○). Theoretical curves derived from the polymerisation kinetics shown in (A) after eq. [3] (– – –) and after equation [9] (———).

state of GTP hydrolysis obtained in the previous experiment after the addition of 300 μM GTP at the polymerisation plateau.

4.2. Correlation between the kinetics of GTP hydrolysis and of tubulin self assembly

Since the experiment previously described suggested that the tubulin GTPase activity was depending on the free GTP concentration, we tried to see if the same conclusions could be drawn from an analysis of the polymerisation kinetics.

Phosphocellulose purified tubulin (c_{tot} = 22 μM) which contained a NDP kinase activity and one GDP on its "E" site (see Materials and Methods) was induced to polymerise in RBMG buffer in the presence of 500 μM ATP or 300 μM GTP. As we mentioned in the previous chapter, phosphorylation of GDP into GTP by the system ATP-NDP kinase and the high affinity of GTP for the tubulin dimer allowed to work at concentration of tubulin-GTP maximal but free GTP concentration very low. In the second case, polymerisation in the presence of 300 μM GTP, the tubulin-GTP concentration was also maximal but the free GTP concentration was relatively large (278 μM, before starting the polymerisation reaction). The turbidity curves obtained are presented in figs. 2A and 3A. After 50 min at 37°C, the microtubules were centrifuged out. A concentration of 10 μM polymerised and 12 μM unpolymerised tubulin was obtained in both cases. Since the increment of optical density expressed in arbitrary unit of optical density (uDO) obtained after 50 min was equal to 0.58 uDO we made the assumption that, all over the turbidity curve, 0.058 uDO increment was representing 1 μM polymerised tubulin. Such an assumption is justified in the range of protein concentration considered here. However it has been shown that at higher protein concentration, the increment in optical density is not any more a simple linear function of the quantity of polymerised tubulin [15]. It was also assumed that the critical concentration ($\bar{c}_1$) was equal to 12 μM. The turbidity curves give the variation of polymerised tubulin, c_w, as a function of time, from which $c_1(t)$ can easily be deduced: $c_1(t) = c_{tot} - c_w(t)$. The values $c_1(t)$ and $dc_1(t)/dt$ were determined every 30 s and $k_{1+}c_p(t)$ was calculated from these values according to eq. (4) for both polymerisation kinetics. The functions were then averaged and plotted on figs. 4A and B. These graphs depict the variation of the number of microtubules, c_p, during the course of polymerisation. We see that c_p increases very fast during the first stages of the polymerisation reaction. It goes through a maximum after which it decreases first rapidly then slowly until it appears to reach a constant value. The value of this contant is difficult to obtain by calculation since, beyond 12 to 14 min, $dc_1(t)$ and $(c_1(t) - \bar{c}_1)$ become too small to be evaluated with a sufficient accuracy. But this value can also be obtained by plotting the function $\ln c_1(t)$ as a function of time. The slope of this function gives $k_{1+}c_p$ according to eq. (2) which is valid if c_p does not depend on time. At time between 12 to 25 min $k_{1+}c_p$ could be evaluated to be of the order of 0.13 min^{-1} for the polymerisation in the presence of ATP and 0.115 min^{-1} for the polymerisation in the presence of GTP.

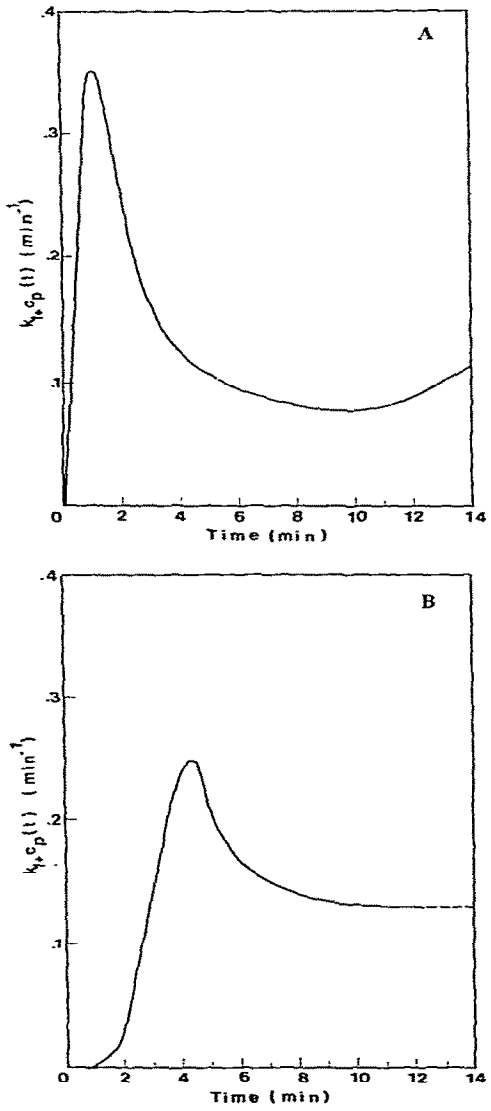


Fig. 4. (A). Variation of $k_{1+}c_p(t)$ during the course of polymerisation of tubulin calculated from the kinetics of polymerisation shown in fig. 2(A) after eq. (4). (B). Variation of $k_{1+}c_p(t)$ during the course of polymerisation of tubulin calculated from the kinetics of polymerisation shown in fig. 3(A) after eq. (4).

The time course of inorganic phosphate release was then calculated by replacing the integrales over the eqs. (3) and (9) by a sum of intervals of 30 s width. It was also determined experimentally. Eq. (9) could be calculated by using the experimental value of 16 μM for k'_+/k_{1+} (see previous chapter). We have plotted on the same graph the computed and the experimental curves in both cases, polymerisation in the presence of GTP (fig. 2B) and polymerisation in the presence of ATP and a stoichiometric amount of GDP to tubulin (fig. 3B). It appears clearly that the experimental points are fitted quite well by the second model "GTP hydrolysis and exchanges at the microtubule ends" eq. (9) for the polymerisation in the presence of 300 μM GTP. In the second case, on the contrary, the experimental points are fitted much better by eq. (3). That is, in this case, the rate of GTP hydrolysis is limited by the turnover of tubulin-GTP at the microtubule ends.

5. Discussion

The previous observation that the steady state rate of GTP hydrolysis of microtubule preparations was proportional to the number of microtubule ends suggested that the tubulin GTPase activity was located at the ends of the microtubules [13]. We assumed in this paper that such a conclusion was still holding when the tubulin preparation contained a mixture of microtubules and sheets. Indeed I have noticed that changing the relative proportion of sheets and microtubules at constant polymer concentration, by changing for instance the Mg^{2+} concentration, did practically not change the steady state rate of GTP hydrolysis (unpublished result). This observation suggests that the edges of the tubulin sheets are not involved in GTPase activity.

The time course of inorganic phosphate release has been derived from the kinetics of tubulin dimer incorporation into the microtubules. Two different mechanisms have been considered: (a) GTP hydrolysis occurs as a tubulin-GTP complex polymerises into the end of a microtubule; (b) GTP hydrolysis is limited by the GTP hydrolysis and exchange at the end of the microtubule.

The kinetics of inorganic phosphate release during polymerisation in excess free GTP is fitted well by the second model. That is, when there is free GTP available in the medium, GTP hydrolysis and exchange occurs

at the microtubule ends independently of the tubulin-GTP polymerisation reaction. This result indicates that the "E" sites on the microtubule ends have remained available for nucleotide exchange contrary to the "E" sites on the tubulin subunits inside the microtubules. Under these experimental conditions, the variation of the rate of GTP hydrolysis during the course of polymerisation follows exactly the variation of the number of microtubules. The variation of the number of microtubules derived from the kinetics of polymerisation in excess free GTP shows that the number of microtubules goes through a maximum at early time (in the present case, one minute after induction of the polymerisation reaction). This maximum introduces an inflexion point into the kinetics of GTP hydrolysis. This inflexion point however is not very much pronounced so that it is difficult to affirm its existence from the experimental data only, due to technical limitations. Calculation of the variation of the number of microtubules was deduced from an analysis of the turbidity curves. In this analysis, it was assumed that the light scattering intensity was proportional to the concentration of tubulin incorporated into microtubules all over the turbidity curve. In fact this approximation does not hold during the first stages of the polymerisation reaction when the microtubules are shorter than the wavelength of the incident light. To turn out the difficulty, we could have adjusted the calculated and experimental curves at the time when the approximation starts holding and tried to fit the curves after that time. This however was not necessary to do since the deviations between the theory and the experiment were not sufficient to justify an adjustment.

The kinetics of inorganic phosphate release during polymerisation in the presence of ATP and a stoichiometric amount of GDP to tubulin is fitted well also by a limiting case of the second model, in which the concentration of GTP in the medium is low compared to the dissociation constant of the microtubule end-GTP complex but still high compared to the dissociation constant of the tubulin-GTP complex. The good fit obtained implies that the dissociation constant of the tubulin-GTP complex is much lower than the dissociation constant of the microtubule end-GTP complex. An estimation of the ratio between the two constants can be given. Call $K_{d,T,GTP}$ and $K_{d,M_n,GTP}$ the respective dissociation constants of the tubulin-GTP (T-GTP) and microtubule end-GTP (M_n-GTP) complexes

$$K_{d,T,GTP} = \frac{T \cdot GTP}{T\text{-}GTP}, \qquad K_{d,M_n,GTP} = \frac{M_n \cdot GTP}{M_n\text{-}GTP}$$

$$\frac{K_{d,T,GTP}}{K_{d,M_n,GTP}} = \frac{T}{T\text{-}GTP}\,\frac{M_n\text{-}GTP}{M_n}$$

It is a reasonable assumption to say that the free GTP concentration is high compared to $K_{d,T,GTP}$ when [GTP] = 10 $K_{d,T,GTP}$ and that it is low compared to $K_{d,M_n,GTP}$ when [GTP] = $\frac{1}{10}K_{d,M_n,GTP}$, that is T/T-GTP = $\frac{1}{10}$ and M_n/M_n-GTP = 10. Therefore the dissociation constant of the microtubule end-GTP complex should be at least 100 times higher than the dissociation constant of the tubulin-GTP complex. Since $K_{d,T,GTP}$ has been estimated between 10^{-6} to 10^{-8} mol ℓ^{-1}, $K_{d,M_n,GTP}$ could be comprise between 10^{-4} to 10^{-6} mol ℓ^{-1}. Since 300 μM total GTP concentration gives a maximal steady state rate of GTP hydrolysis, a maximal value for $K_{d,M,GTP}$ can be put equal to 30 μM. The limiting case of the second model in which the free GTP concentration is low compared to $K_{d,M_n,GTP}$ is theoretically identical to the first model. The analogy is easily understandable. If there is no free GTP available for hydrolysis and exchange at the microtubule ends, eventually the GTP will be brought to the microtubule end only through the association of a tubulin-GTP complex and this situation will be identical to that of the first model. But, if the first model had been the general one, one would have expected no effect at all of free GTP added on the tubulin GTPase activity contrary to what happens.

The present result that the microtubule end "E" sites remain exchangeable is consistent with the reported observation that the microtubules generally bear less than one "E" site GDP per polymerised tubulin dimer [2]. If indeed, the guanine nucleotides can dissociate from "E" sites at the microtubule ends, this site will eventually become blocked through the addition of a new tubulin-GTP complex before it has been occupied again.

The polymerisation of monomeric actin into filaments is associated with an ATP hydrolysis [28]. Recently, in a study to which the present study is analoguous, Wegner [29] proposed that "ATP splitting and exchange was mainly connected with the association of actin protomers at the end of actin filaments". He noticed however some systematic deviations be-

tween the theory and the experiments. It might be that in the case of actin polymerisation also inorganic phosphate release is limited by the rate of nucleotide hydrolysis and exchange at the end of the polymers and not by the rate of protomer association.

Notice that we included in the model the assumption that a tubulin-GTP complex at a microtubule end cannot dissociate before the GTP on its "E" site had been hydrolysed. Without this assumption, the model would not fit the experimental data. It has been reported that the microtubules polymerised in the presence of a non-hydrolysable analog of GTP, (GMP-PNP), do not depolymerise at very low concentration up to 1 h after dilution [30]. The authors then suggested that hydrolysis of GTP would promote the depolymerisation of the microtubules. This observation is consistent with our working hypothesis that a tubulin-GTP complex cannot dissociate from the microtubule end.

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Appendix

$$\mathrm{d}[\mathrm{M}_n\text{-GTP}](t)/\mathrm{d}t = k_{1+}c_\mathrm{p}(t)c_1(t) - k'_+[\mathrm{M}_n\text{-GTP}]. \tag{10}$$

Call $[\mathrm{M}_n\text{-GTP}](t) = c(t)$ and $k_{1+}c_\mathrm{p}(t)c_1(t) = f(t)$, then (10) becomes:

$$\mathrm{d}c(t)/\mathrm{d}t = f(t) - k'_+c(t). \tag{14}$$

Put

$$c(t) = \mathrm{e}^{-k'_+t}y(t). \tag{15}$$

Replace eq. (15) in (14):

$$\frac{\mathrm{d}c(t)}{\mathrm{d}t} = -k'_+\mathrm{e}^{-k'_+t}y(t) + \frac{\mathrm{d}y(t)}{\mathrm{d}t}\,\mathrm{e}^{-k'_+t}$$

$$= -k'_+\mathrm{e}^{-k'_+t}y(t) + f(t). \tag{16}$$

We obtain:

$$\mathrm{d}y(t)/\mathrm{d}t = f(t)\mathrm{e}^{-k'_+t}. \tag{17}$$

$\mathrm{d}y(t)/\mathrm{d}t$ varies like $\mathrm{e}^{k'_+t}$ if k'_+ is large. Therefore the solution of eq. (5) is:

$$y(t) = (1/k'_+)\mathrm{e}^{k'_+t}f(t). \tag{18}$$

From eqs. (18) and (15) we obtain:

$$c(t) = \frac{1}{k'_+}f(t) = \frac{k_{1+}}{k'_+}c_\mathrm{p}(t)c_1(t). \tag{19}$$

It can be shown that the condition k'_+ large is satisfied. Indeed, experimental values for $k_{1+}c_\mathrm{p}$ at the turbidity plateau have been reported equal to 0.11 and 0.13 min^{-1} (see sect. 4). At the polymerisation plateau also, the average length of the microtubules is of the order of 4 μm. A 4 μm length microtubule contains 6500 tubulin subunits, therefore a 10 μM polymerized tubulin concentration corresponds to a 1.5×10^{-3} μM microtubule concentration (c_p). An order of magnitude for the constants k_{1+}, k'_+ and k_{2-} is then:

$$k_{1+} = \frac{0.11}{c_\mathrm{p}}\ \mathrm{min}^{-1} \approx 7 \times 10^7\ \mathrm{mol}^{-1}\ \ell\ \mathrm{min}^{-1}$$

$$k_{2-} = k_{1+}\bar{c}_1 \approx 7 \times 10^7 \times 10^{-5}\ \mathrm{min}^{-1} = 7 \times 10^2\ \mathrm{min}^{-1}$$

$$k'_+/k_+ \approx 16\ \mu\mathrm{M}, \qquad \text{(experimental value)}.$$

Then

$$k'_+ \approx 16 \times 10^{-6}\ k_{1+} \approx 10^{-3}\ \mathrm{min}^{-1}.$$

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