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GTPase ACTIVITY OF THE TUBULIN-COLCHICINE IN RELATION WITH TUBULIN-TUBULIN INTERACTIONS.

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Summary: The GTPase activity of the tubulin-colchicine complex has been studied at different tubulin-colchicine concentrations. The specific activity was found to decrease at low concentrations. Several hypothesis accounting for this observation have been discarded, and the activation via collisions between two molecules of tubulin has been considered as a possible model explaining the origin and observed concentration dependence of the GTPase activity. The activation of tubulin-colchicine by unliganded tubulin or tubulin-podophyllotoxin has been investigated within this model which emphasizes the connection between some specific tubulin-tubulin interactions and the conformation of the exchangeable nucleotide site on tubulin.

INTRODUCTION

Tubulin assembly in microtubules is accompanied by the hydrolysis of GTP and subsequent sequestration of GDP at the "exchangeable" site (1). GTP hydrolysis has been shown to occur in a kinetic first order process following the assembly process but kinetically uncoupled from it, which suggested the hypothesis of a conformation change of the tubulin molecule once incorporated in the polymer which would trigger GTP hydrolysis (2). Binding of colchicine to tubulin inhibits microtubule assembly (3) and induces a low but significant GTPase activity on tubulin (4). This observation and the fact that the colchicine site is masked on microtubules (5) and exposed on linear polymers of tubulin (6) and of tubulin-vinblastine (7) were suggestive of a tight connection between the colchicine site and one of the two lateral interaction areas in the tubulin molecule (4, 8). In this hypothesis colchicine binding would induce the lateral area conformation change triggering GTP hydrolysis. In the aim of elucidating the origin and the nature of the connection between tubulin-tubulin interactions and the events at the nucleotide site, the concentration dependence of the GTPase activity of the tubulin-colchicine complex has been investigated. The results support a model in which GTP is hydrolyzed via an activated complex resulting from the collision between two molecules of tubulin-colchicine. The implication of these new data in anterior results is discussed.

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MATERIALS AND METHODS

Tubulin was purified from fresh pig brains through three assembly-disassembly cycles according to Shelanski et al. (9), followed by phosphocellulose chromatography according to Weingarten et al. (10). Pure tubulin was stored at -70°C in 50 mM MES pH 6.6, 3.4 M glycerol, 0.25 mM $MgCl_2$ and used within a few weeks. Before use, tubulin was chromatographied through Sephadex G25 (PD10, Pharmacia) equilibrated in 0.1 M MES pH 6.6, 1 mM EGTA, 0.5 mM $MgCl_2$. All experiments were performed in this buffer.

Colchicine binding was performed at 37°C using the activated charcoal adsorption assay described by Sherline (11). Tubulin concentration in the binding assay was 10-30 µM. The extent of binding was routinely 0.9 to 1.0 mole of colchicine bound per tubulin dimer, from UV-visible spectroscopic determination using a value of 16,000 M^{-1} cm^{-1} for the extinction coefficient of colchicine at 350 nm, and from radioactivity measurement using [^{3}H]-colchicine (Amersham).

The time course of GTP hydrolysis on the tubulin-colchicine complex was monitored at 37°C by measurement of [^{32}P] liberated from γ-[^{32}P]-GTP, at a number of time intervals, according to Avron (12).

Sedimentation-diffusion equilibrium measurements of the tubulin-colchicine complex were carried out in a Beckman model E ultracentrifuge equipped with an electronic speed regulation and a RTIC temperature control. Centrifugation was run at 20,000 rpm and at 30°C, in a type AV.D. rotor.

RESULTS AND DISCUSSION

1. Concentration dependence of the specific GTPase activity of the tubulin-colchicine complex

Tubulin-colchicine GTPase activity was assayed at a number of concentrations of tubulin-colchicine in the 1 µM-20 µM concentration range. At all concentrations, the time courses of Pi liberation were linear up to 30-60 min reaction which allowed an accurate measurement of the initial rate of GTP hydrolysis. Fig. 1 shows that at concentrations lower than $\sim$ 10 µM, the specific GTPase activity of the tubulin-colchicine complex was no more constant and exhibited a significant decrease, extrapolating to zero at zero tubulin-colchicine concentration. Above 10 µM, the specific activity of tubulin-colchicine was constant and the observed turnover was 0.1 min^{-1}, a value in agreement with Saltarelli and Pantaloni (8). Several possibilities could account for this observation and the following ones have been examined :

α) The dissociation of colchicine from its site upon dilution of tubulin-colchicine could cause a decrease of the specific activity at low concentration. This possibility was eliminated because the same curve as in Fig. 1 was obtained when the assays were performed in the presence of a saturating (100 µM) concentration of colchicine.

β) The possibility of an inactivation of the tubulin-colchicine due to a dissociation into α and β subunits upon dilution was examined in

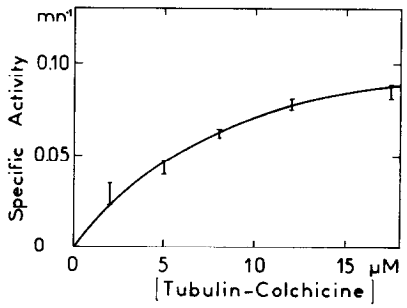


Figure 1. Plot of the specific GTPase activity of tubulin-colchicine complex versus tubulin-colchicine concentration. GTP hydrolysis was assayed as described in Methods in the presence of 150 µM γ-[^{32}P]GTP. Colchicine binding was 1 mole colchicine per mole of tubulin. Bars represent the uncertainty in the GTPase activity measurements. The curve is theoretical and calculated according to equation (3) using the values $k_+ = 0.015\ \mu M^{-1}\ min^{-1}$ and $k_h \times \frac{k_+}{k_-} = 0.1\ min^{-1}$.

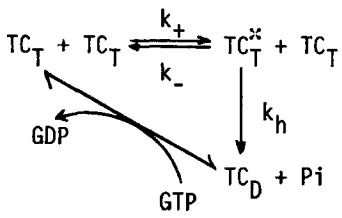
the ultracentrifuge : no dissociation appeared in the concentration range in which the change in activity was observed, and in a low-speed equilibrium experiment, a constant slope of the plot Ln C versus r^2 corresponding to a value of M_r of 112,000 was observed, in agreement with Detrich et al. (13).

γ) For the same reason as above, the phenomenon could not be explained by the dissociation of an active dimer $(\alpha\beta)_2$ into an inactive $\alpha\beta$ species of the tubulin-colchicine complex.

δ) It was further checked that the observed decrease in specific activity was not time dependent but was a true equilibrium phenomenon : A sample was assayed in which the tubulin-colchicine concentration was 11 µM and the turnover rate 0.066 min^{-1}. The sample was first diluted to 3 µM and assayed. A value of 0.027 min^{-1} was found for the turnover rate. The diluted sample was then concentrated to 7 µM using an Amicon Ultrafiltration cell equipped with a Diaflo PM30 membrane and showed a turnover rate of 0.050 min^{-1}. These values were in agreement with the expected ones from the plot of Fig. 1, and show that the observed variation results from a rapidly reversible equilibrium.

2. A possible model which could account for the data involves the collisions between two molecules of tubulin-colchicine (TC) in solution which would give rise to an intermediary transient (TC-TC), in concentrations too weak to be detectable. The collision would generate an activated molecule TC^* able to hydrolyze GTP with a first order rate constant k_h. The activated molecules could alternatively reversibly desactivate into TC in another collision. This model is identical to the one proposed by Lindemann (14) and is

described by the following scheme :



in which TC_T and TC_D represent the tubulin-colchicine complex having GTP and GDP bound respectively, and k_+ and k_- are the activation and desactivation rate constants. The exchange of nucleotides on TC is a fast process at 37°C.

This model is described by the following equations :

$$(1)\quad \begin{cases} \dfrac{d[TC_T^*]}{dt} = k_+[TC_T]^2 - k_-[TC_T][TC_T^*] - k_h[TC_T^*] \\ \dfrac{d[Pi]}{dt} = k_h[TC_T^*] \end{cases}$$

At steady state, $\dfrac{d[TC^*]}{dt} = 0$ and (1) resolves into :

$$(2)\quad [TC_T^*] = \frac{k_+[TC]^2}{k_-[TC] + k_h}$$

and the rate of Pi liberation is :

$$(3)\quad v = \frac{d[Pi]}{dt} = k_h\, k_+ \frac{[TC]^2}{k_-[TC] + k_h}$$

Equation (3) shows that when the concentration of TC is much lower than $\frac{k_h}{k_-}$, the specific GTPase activity $\frac{v}{[TC]}$, approximates to $k_+[TC]$ whereas at high concentrations, it becomes independent of [TC] and equal to $k_h \times \frac{k_+}{k_-} = k_{cat}$. The data shown in fig. 1 fit well with this model and the following values can be derived from the plot of $\frac{v}{[TC]}$ versus [TC] :

$$k_+ = .015\ \mu M^{-1}\ min^{-1}$$

$$k_{cat} = 0.1\ min^{-1} = k_h \times \frac{k_+}{k_-}\ ,\ \text{giving}\ \frac{k_h}{k_-} = 6.7\ \mu M$$

3. The model predicts that at low tubulin-colchicine concentration, the number of activating collisions could be increased by the addition of increasing amounts of unliganded tubulin which has no GTPase activity by it-

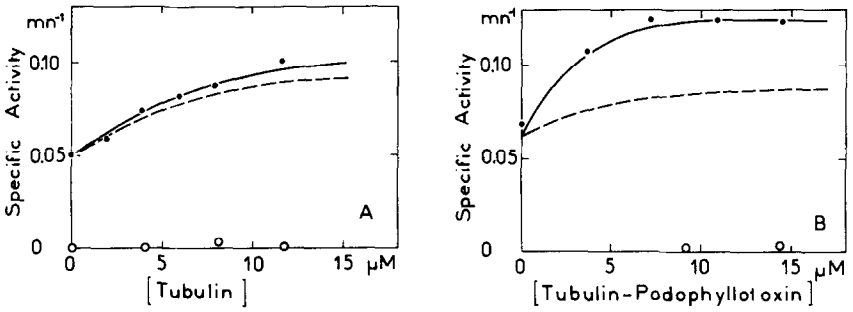
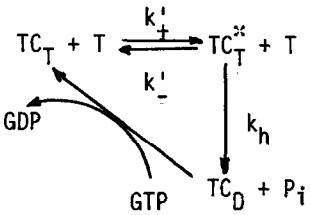


Figure 2. Efficiency of unliganded tubulin (A) and tubulin-podophyllotoxin (B) in the activation of GTP hydrolysis on the tubulin-colchicine complex. Increasing concentrations of unliganded tubulin were added to a constant concentration of tubulin-colchicine (5.5 µM in A, 6.2 µM in B). In B, 100 µM podophyllotoxin was present in the reaction mixture. Full circles : the specific activity of tubulin-colchicine is plotted versus the concentration of added unliganded tubulin (A) or tubulin-podophyllotoxin (B). Open circles : GTPase activity measured in control samples in which no tubulin-colchicine was present. The dashed line represents the above observed change in specific activity upon increasing tubulin-colchicine concentration (cf. fig. 1).

self, to a constant low amount of tubulin-colchicine. This experiment was performed and the results shown in fig. 2 indicate that the expected increase in the specific activity of tubulin-colchicine was observed. No such increase was obtained when bovine serum albumin was added to tubulin-colchicine instead of tubulin. The kinetic parameters describing the activating collisions between tubulin-colchicine (TC) and tubulin (T) can be derived from the data within the additive scheme :



In the presence of T, equation (1) is modified and becomes :

$$(4) \quad \frac{d[TC^*]}{dt} = k_+[TC]^2 + k_+[TC].[T] - [TC^*](k_-[TC] + k'_-[T]) - k_h[TC^*]$$

and the rate of P_i liberation now is :

$$(5) \quad \frac{dPi}{dt} = k_h[TC]\,\frac{k_+[TC] + k'_+[T]}{k_-[TC] + k'_-[T] + k_h}$$

Equation (5) indicates that when the concentration of T is increased at a constant concentration of TC, the limit specific activity reached at

infinite T concentration is $k'_{cat} = k_h \frac{k'_+}{k'_-}$. This utrnover rate can be different from the $k_{cat} = k_h \frac{k_+}{k_-}$ obtained above upon increasing TC concentration. The plot shown in fig. 2A indicates that when unliganded tubulin was added to tubulin-colchicine the same activation of tubulin-colchicine GTPase activity was obtained as when tubulin-colchicine concentration was varied. This result indicates that $\frac{k'_+}{k'_-} = \frac{k_+}{k_-}$ i.e. the collisions between TC and T are as efficient as between two molecules of TC in promoting GTPase activity of the tubulin-colchicine complex.

The same experiment was performed using tubulin-podophyllotoxin instead of native tubulin. This was realized by adding 100 µM podophyllotoxin to the reaction medium containing TC and T, conditions under which all the initially unliganded tubulin becomes in the form of tubulin-podophyllotoxin complex which has no GTPase activity by itself (4). In this case a stronger stimulation was obtained, and a higher specific activity at high tubulin-podophyllotoxin concentrations, indicating that $\frac{k'_+}{k'_-} > \frac{k_+}{k_-}$. In other words, a higher steady-state amount of the activated species $\overline{TC}^*$ is formed through collisions between tubulin-colchicine and tubulin-podophyllotoxin than through the homologous collisions between two molecules of TC.

In conclusion, the collision model emphasizes the role of some specific tubulin-tubulin interactions in triggering GTP hydrolysis, which has been evoked in connection with tubulin assembly. The colchicine site appears in close interaction with the GTP site and thus may also be located on the β subunit as suggested by genetic data (15, 16).

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