Roberts, J. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 247–271, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Rosenberg, M., & Court, D. (1979) *Annu. Rev. Genet. 13*, 319–353.
Rosenberg, M., Court, D., Shimatake, H., Brady, C., & Wulff, D. L. (1978) *Nature (London) 272*, 414–422.
Shen, C.-K. J., Wiesehahn, G., & Hearst, J. E. (1976) *Nucleic Acids Res. 3*, 931–951.
Spindler, S. R., Deuster, G. L., D'Alessio, J. M., & Paule, M. R. (1978) *J. Biol. Chem. 253*, 4669–4675.
Stauffer, G. V., Zurawski, G., & Yanofsky, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4833–4837.
Tinoco, I., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M., & Gralla, J. (1973) *Nature (London), New Biol. 245*, 40–41.
Youvan, D. C., & Hearst, J. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3751–3754.
Zain, B. S., Weissmann, S. M., Lebowitz, P., & Lewis, A. M., Jr. (1973) *J. Virol. 11*, 682–693.
Zain, B. S., Weissmann, S. M., Dhar, R., & Pan, J. (1974) *Nucleic Acids Res. 1*, 577–594.
Zurawski, G., Brown, K., Killingly, D., & Yanofsky, C. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4271–4275.
Zurawski, G., Elseviers, D., Stauffer, G. V., & Yanofsky, C. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5988–5992.

Kinetic Analysis of Guanosine 5′-Triphosphate Hydrolysis Associated with Tubulin Polymerization†

Marie-France Carlier* and Dominique Pantaloni

ABSTRACT: The correlation between the time courses of pure tubulin assembly and accompanying guanosine 5′-triphosphate (GTP) hydrolysis has been studied at different tubulin concentrations in the range where the rate of assembly varies with a strong cooperativity. One GTP molecule was found hydrolyzed per molecule of tubulin dimer incorporated in the microtubule. This hydrolysis was not strictly coupled to polymerization and occurred in a subsequent step. Consequently, in the first stages of assembly, tubulin–GTP complex is the transient major constituent of microtubules. Kinetic data of GTP hydrolysis have been treated within a model of two consecutive first-order reactions:

$$[\text{tubulin–GTP}]_{\text{free}} \xrightarrow{k_1} [\text{tubulin–GTP}]_{\text{MT}} \xrightarrow{k_2} [\text{tubulin–GDP}]_{\text{MT}} + [\text{P}_i]$$

GTP hydrolysis proceeded at an intrinsic rate $k_2 = 0.25\ \text{min}^{-1}$ independent of tubulin concentration. Simultaneous measurements of polymerization, GTPase activity, and incorporation of [^{3}H]GTP followed by unlabeled GTP chase indicated that before its hydrolysis GTP bound to microtubules was exchangeable while after hydrolysis GDP remained locked in the E site. The possibility is discussed that after assembly tubulin undergoes a conformation change which could trigger GTP hydrolysis and sequestration of GDP.

The complete understanding of the mechanism and regulation of microtubule assembly requires a thorough investigation of the relationship between tubulin polymerization and accompanying guanosine 5′-triphosphate (GTP) hydrolysis.

The question has been considered by many authors, and definite agreement has been reached on several points. It is now established that GTP is hydrolyzed only at the "exchangeable" (E) site of tubulin during polymerization, P_i is released in the medium, and GDP remains blocked in the microtubule (Jacobs et al., 1974; Kobayashi, 1975; Weisenberg et al., 1976; David-Pfeuty et al., 1977). In the absence of free GTP in the medium, polymerization of the preformed tubulin–GTP complex occurs concomitant with the hydrolysis of one molecule of GTP per molecule of tubulin in the microtubule (MacNeal & Purich, 1978a).

Hydrolysis of GTP is involved in two aspects of microtubule assembly, namely, the mechanism and energetics of tubulin–tubulin interaction and the regulation of the equilibrium or steady-state microtubules $\rightleftarrows$ tubulin. Indeed, the irreversible process of GTP hydrolysis at steady state has been the support for a "head-to-tail" polymerization mechanism proposed by Margolis & Wilson (1978) and first explicited by Wegner (1976) in the case of actin, another cytosqueleton contractile protein exhibiting the similar feature of nucleotide hydrolysis accompanying assembly. Puzzling findings raised the problem of the role of and requirement for GTP hydrolysis in microtubule assembly: microtubules can be obtained and stablized in the presence of large amounts of nonhydrolyzable analogues of GTP (Arai & Kaziro, 1976; Penningroth & Kishner, 1977; Weisenberg & Deery, 1976) and have almost the same equilibrium dissociation constant as microtubules formed in the presence of GTP (Karr et al., 1979). It was demonstrated in this laboratory (Carlier & Pantaloni, 1978) and confirmed by others (Karr et al., 1979; Zackroff et al., 1980) that GDP allowed elongation of microtubules if not nucleation and that the equilibrium dissociation constant of microtubules stabilized in the presence of GDP was only twice larger than the one obtained in the presence of GTP.

Use of 8-azido-GTP as a photoaffinity probe indicated that hydrolysis occurred on the β subunit of tubulin concurrently with polymerization (Geahlen & Haley, 1977, 1979). Although experiments performed with drugs inhibiting assembly (David-Pfeuty et al., 1979) show that the GTPase activity of tubulin can be induced in the absence of polymerization, it is nevertheless well established that during microtubule as-

† From the Laboratoire d'Enzymologie, C.N.R.S., 91190 Gif-sur-Yvette, France. *Received July 29, 1980.*

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sembly GTP hydrolysis is dependent on polymerization. However, the exact nature of the connection between the two processes remains unknown.

In this work are simultaneously investigated the time courses of tubulin polymerization and accompanying GTP hydrolysis at different tubulin concentrations. Since it has already been shown that the rate of tubulin polymerization varied cooperatively with tubulin concentration, this approach was expected to provide insights in the relation between GTP hydrolysis and microtubule assembly. A preliminary report of these results was presented (Carlier & Pantaloni, 1980a,b).

Materials and Methods

Reagents. 2-(*N*-Morpholino)ethanesulfonic acid (Mes) was purchased from Calbiochem, guanosine 5′-triphosphate trilithium salt was from Boehringer, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and poly(ethylenimine)–cellulose (200 mesh) were from Sigma, and DEAE-Sephadex A50 was from Pharmacia; glycerol and all salts used in buffers were of analytical grade from Merck. [^{3}H]- and [γ-^{32}P]guanosine 5′-triphosphate were purchased from Amersham.

Tubulin Purification. Tubulin was purified from fresh pig brain by three consecutive cycles of assembly–diassembly, according to Shelanski et al. (1973), followed by gel chromatography through DEAE-Sephadex A50, according to Murphy et al. (1977). After elution of microtubule-associated proteins (MAPs) with 0.3 M NaCl in 0.1 M Mes buffer, pH 6.6, containing 0.5 mM $Mg(CH_3CO_2)_2$, 1 mM EDTA, and 50 μM GTP, tubulin was eluted with 0.8 M NaCl in the same buffer and concentrated by ammonium sulfate precipitation and centrifugation at 0 °C for 15 min at 25000 *g* in a Sorvall RC 2B centrifuge. The pellet was dissolved in 0.05 M Mes buffer containing 0.25 mM $Mg(CH_3CO_2)_2$, 0.5 mM EGTA, 50 μM GTP, and 3,4 M glycerol and dialyzed at 4 °C for 15 h against the same buffer. The tubulin solution thus obtained was stored at −70 °C and routinely used within the 2–3 following weeks. This preparation was pure and particularly was free of nucleoside diphosphokinase activity (Jacobs & Huitorel, 1979). Protein concentration was estimated by the method of Lowry et al. (1951) with correction for the excess coloration of tubulin in this test as compared with bovine serum albumin (David-Pfeuty et al., 1977).

Polymerization Measurements. Polymerization of tubulin was studied in P buffer consisting of 0.05 M Mes, pH 6.6, 6 mM $MgCl_2$, 0.5 mM EGTA, 3.4 M glycerol, and varying amounts of GTP, which is a modification of the buffer used by Lee & Timasheff (1975).

Tubulin self-assembly was monitored by turbidimetry at 350 nm by using a Beckman Acta V recording spectrophotometer equipped with a thermostated 100-μL and 0.5-cm light path cell connected with a slow T-jump apparatus. The observed turbidity values were corrected as described (Carlier & Pantaloni, 1978) in order to obtain data directly proportional to the amount of polymer formed.

Microtubules could be rapidly separated from the free tubulin dimer solution (in less than 40 s) by centrifugation at 160000*g* for 2 min in a Beckman airfuge with a prewarmed rotor at 37 °C and a warm air circulation, maintaining the rotor temperature at least at 30 °C in order to avoid depolymerization of microbutules during the centrifugation. This technique permitted the direct measurement of the weight of microtubules at different times in the course of polymerization.

Incorporation of Labeled Nucleotides in Microtubules. Aliquots of 125 μL of microtubule solutions containing radioactive nucleotides were centrifuged at 160000*g* in the airfuge in polyethylene microtubes. The supernatant was pipetted off the tubes, and the tube walls and pellet surface were rinsed 3 times with warm unlabeled polymerization buffer containing 30% sucrose and then dissolved by incubation with 50 μL of formic acid for 15–20 min. The radioactivity contained in the pellets was then determined by counting the whole tube in 10 mL of Aquasol 2 (New England Nuclear) in a Packard Tricarb Model 3310 liquid scintillation spectrometer. A contamination control sample was always run in which radioactivity was added to the same amount of polymer just before the centrifugation, in the presence of a large amount of unlabeled nucleotide, in order to avoid any incorporation of label in the microtubules. The extent of contamination varied between 0.5% and 1.5% of the total radioactivity present in the assay, according to the weight of polymer centrifuged, and its value was subtracted from the radioactivity content of the parallel sample. A correction was made for 20% quenching in the countings of the pellets due to formic acid. The specific radioactivity of the supernatant was also determined.

Determination of the Concentration and Analysis of Nucleotides. Knowledge of the exact concentration of free and exchangeable GTP in the assay is important for the accurate determination of labeled GTP specific radioactivities in the measurements of [γ-^{32}P]GTP hydrolysis and [^{3}H]GDP incorporation in microtubules. The concentration of free and bound (exchangeable and nonexchangeable) guanine nucleotides present in tubulin solutions was determined spectrophotometrically after 4% perchloric acid extraction, using an extinction coefficient of 12400 M^{-1} cm^{-1} at 252 nm. Correction was made for the nonexchangeable GTP present in the extracted nucleotide solution in 1:1 ratio to tubulin. The separation and quantitative estimation of the amount of GDP and GTP were obtained by chromatography of the neutralized nucleotide perchloric extract on poly(ethylenimine)–cellulose and stepwise elution with increasing concentrations of LiCl, using a ISCO UV monitor equipped with 254-nm filters and an absorbance recording chart. The ratio of GDP to GTP present was estimated from the areas under the GDP and GTP peaks in the elution pattern.

GTPase Activity Measurements. The GTPase activity of tubulin during polymerization was monitored by determination of radioactive $^{32}P_i$ liberated during hydrolysis, according to Avron (1960). Typically, 1 mL of a solution of 10–40 μM tubulin in P buffer containing 100–250 μM [γ-^{32}P]GTP was placed at zero time in preheated tubes, and the reaction was followed in a thermostated bath at 37 °C, by pipetting 60-μL aliquots from the reaction mixture every 30–60 s into the hydrochloric solution of ammonium molybdate. A volume of 50 μL of a 20 mM H_3PO_4 solution was added as a carrier, and the extraction of P_i was proceeded with 2-butanol–benzene–acetone–ammonium molybdate (750:750:15:1) solution saturated with water. An aliquot of the organic phase was counted in 10 mL of Aquasol.

Results

(1) Time Course of GTP Hydrolysis during Tubulin Polymerization. Tubulin assembly in P buffer containing 100–200 μM GTP was followed by turbidimetry at 37 °C, and the liberation of P_i was measured in parallel. Figure 1 shows the evolution of both parameters with time. When tubulin was polymerized at a concentration of 3 mg/mL, the formation of 95% of the microtubules was achieved within 2 min. On the other hand, the rate of GTP hydrolysis slowed down and reached a stable steady-state rate only about 20–25 min after the reaction was started. The extrapolation of the linear

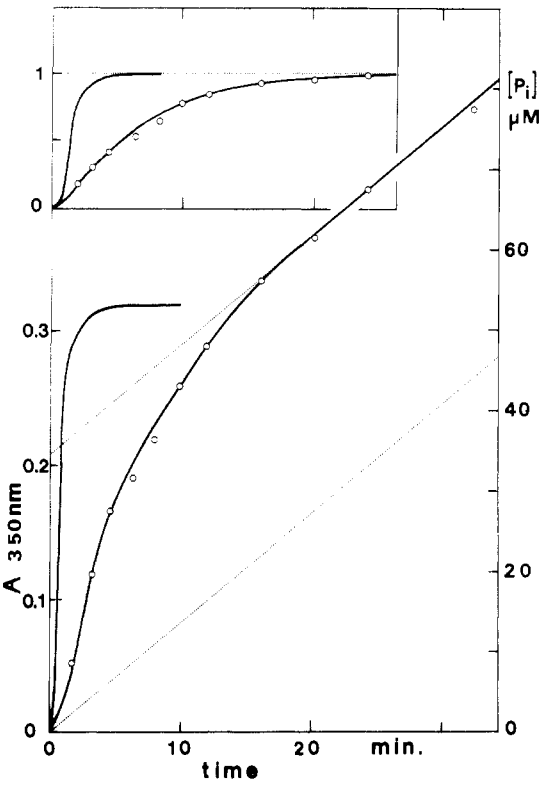

FIGURE 1: Time course of the formation of microtubules and accompanying GTP hydrolysis. Tubulin, 34.5 μM, was polymerized at 37 °C in P buffer containing 198 μM GTP. (Full solid line) Recorded turbidity at 350 nm; (O) amount of $^{32}P_i$ liberated upon GTP hydrolysis. Inset: Same data, normalized with respect to the final amount of microtubules (30 μM). (Full line) Turbidity measurement; (O) burst of P_i production during assembly obtained after subtraction of the steady-state GTP hydrolysis from the whole phenomena.

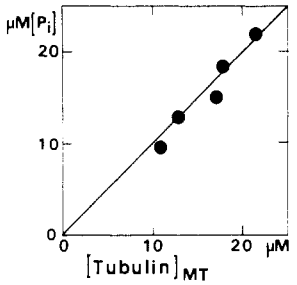

FIGURE 2: Correlation between the amplitude of the burst of GTP hydrolyzed and the amount of tubulin incorporated in the microtubules at equilibrium. Tubulin was polymerized in P buffer in the presence of 151 μM [γ-^{32}P]GTP. Tubulin concentration was varied in the range 1.9–5 mg/mL. The slope is 1 ± 0.1.

portion of the curve at time zero of assembly gave the value of the "burst" of P_i accompanying tubulin polymerization. When tubulin was polymerized at different concentrations, the values found for the burst varied linearly with the concentration of tubulin incorporated in the microtubules, with a slope of 1 ± 0.1 P_i molecule produced per molecule of tubulin polymerized (Figure 2).

The steady-state rate of P_i liberation has been shown to be due to GTP hydrolysis at the ends of microtubules (David-Pfeuty et al., 1977). Previous kinetic studies of microtubule formation have demonstrated that the elongation is a first-order process, which means that the number concentration of elongating species is kept constant during the whole process (Carlier & Pantaloni, 1978). Since the concentration of microtubule ends is constant as soon as elongation begins, the steady-state rate of GTP hydrolysis observed at equilibrium

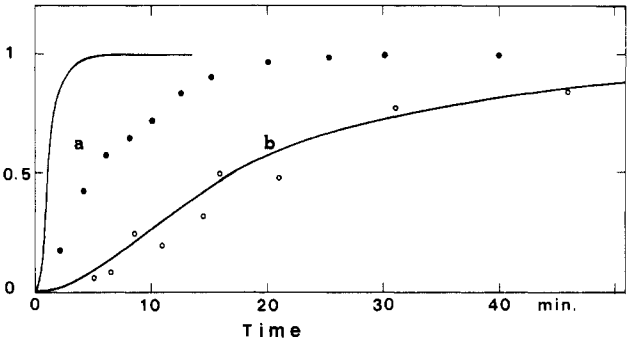

FIGURE 3: Effect of tubulin concentration on the polymerization and concomitant GTP hydrolysis (normalized data). (Full line) Time course of polyermization; symbols represent burst of P_i liberation. Tubulin concentrations were 34.5 (a, ●) and 17.2 μM (b, O).

can be subtracted all along the curve of P_i production. The resulting pre-steady-state phase was a burst of liberation of one molecule of P_i per molecule of tubulin dimer incorporated in the microtubules. Since the turbidity data can be converted into microtubule weight concentration, pending a slight correlation at high tubulin concentrations, both time courses of corrected turbidity evolution and of P_i production in the burst during assembly could be normalized to 1 mol of polymerized tubulin (Figure 1, inset). This representation shows that although GTP hydrolysis is dependent upon tubulin assembly these events are kinetically distinct, and 1 mol of GTP per mole of polymerized tubulin is slowly hydrolyzed after the formation of microtubules.

Evidence has already been shown for a strongly cooperative evolution of the rate of microtubule elongation with tubulin concentration. The correlation between polymerization and GTP hydrolysis kinetics was now studied at different tubulin concentrations. Six tubulin concentrations have been studied, in a range (35–17 μM) where the rate of polymerization varies between 1 and 0.05 min^{-1}. Figure 3 shows typical normalized representations of polymerization and the burst of GTP hydrolysis at two extreme tubulin concentrations of the present range. It clearly appears that the two processes are closer to each other when the polymerization process is slower, i.e., at low tubulin concentration. On the contrary, they seem disconnected at high tubulin concentration. In this case, only 10% of GTP was hydrolyzed when almost 90% microtubules were formed, and the burst was a first-order process having an apparent rate constant of 0.19 min^{-1}, whereas the polymerization rate constant was 0.75 min^{-1}. At intermediary concentrations, the time courses for GTP hydrolysis were complex and could be fitted to a simple exponential.

Two general models can be designed to describe the connection between polymerization and GTP hydrolysis:

first model (cyclic model)

$$MT_n(GDP) + T\text{-}GTP \overset{k_1}{\rightarrow} MT_n\text{-}T\text{-}GTP \overset{k_2}{\rightarrow} MT_{n+1}(GDP) + P_i$$

In this model, the complex T–GTP cannot bind to a microtubule unless it carries GDP only. In this case, polymerization and GTP hydrolysis are kinetically coupled and proceed at the same limiting rate, which is the smaller of k_1 and k_2 at any tubulin concentration. Our data show that this model must be eliminated because it is too restrictive; it seems to account for the data only at low tubulin concentration.

second model (uncoupled model)

$$MT_{m+n}(GDP_m, GTP_n) + T\text{-}GTP \overset{k_1}{\rightarrow} MT_{m+n+1}(GDP_m, GTP_{n+1})$$

$$MT_{m+n}(GDP_m, GTP_n) \overset{k_2}{\rightarrow} MT_{m+1,n-1}(GDP_{m+1}, GTP_{n-1}) + P_i$$

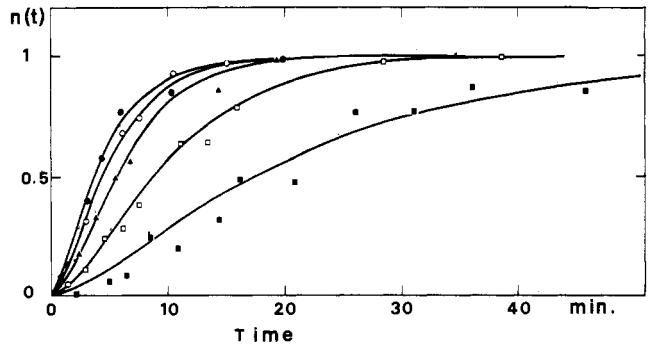

FIGURE 4: Burst of GTP hydrolyzed during tubulin assembly at different tubulin concentrations. $n(t)$ represents the number of moles of P_i produced at each time per mole of tubulin incorporated in the microbutules at equilibrium. The amount of microtubules was measured independently at equilibrium for each tubulin concentration. The curves are theoretical and were obtained by computer adjustment of experimental data (symbols) to eq 1. GTP concentration was 151 μM. Tubulin concentrations (in μM) were the following: (●) 41; (○) 31; (▲) 27; (□) 21.5; (■) 17.4.

In this model, polymerization and GTP hydrolysis are uncoupled and occur with independent rate constants k_1 and k_2. In other terms, in this model, GTP hydrolysis is not a limiting step for polymerization, which can occur faster than GTP hydrolysis. Consequently, in this case, microtubules may have GTP bound. GTP hydrolysis occurs on microtubules as a first-order process with an intrinsic rate constant k_2. The data were analyzed within this model which consists of two consecutive first-order reactions

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

and has been studied by Chien (1948). In the present case, the first reaction (k_1) is the incorporation of the tubulin–GTP complex in the microtubule which has been shown to obey pseudo-first-order kinetics; the second reaction is the hydrolysis of GTP in the microtubule which can be expressed more simply in the following way:

$$[\text{T–GTP}]_{\text{free}} \underset{\longleftarrow}{\overset{k_1}{\rightleftharpoons}} [\text{T–GTP}]_{\text{MT}}$$

$$[\text{T–GTP}]_{\text{MT}} \xrightarrow{k_2} [\text{T–GDP}]_{\text{MT}} + P_i$$

According to this model, hydrolysis of GTP obeys the equation

$$[P_i](t) = [\text{T–GDP}]_{\text{MT}}(t) = \frac{1}{k_1 - k_2}[k_1(1 - e^{-k_2 t}) - k_2(1 - e^{-k_1 t})] \quad (1)$$

Data obtained for the kinetics $P_i(t)$ were fitted to eq 1 for the determination of k_1 and k_2 parameters, using a multiple-regression iterative program working in a Wang 700–720 computer. Figure 4 shows the adjustment obtained for the six tubulin concentrations assayed. In all cases, a satisfactory agreement was found between the values obtained for the apparent polymerization rate constant k_1 from the analysis of the kinetics of inorganic phosphate liberation on the one hand, and from the independent analysis of turbidity pseudo-first-order kinetics. While the values found for k_1 varied cooperatively, as previously observed, from 1 to 0.05 min^{-1} in the investigated range of tubulin concentrations, a constant value of 0.25 min^{-1} independent of tubulin concentration was found for the intrinsic hydrolysis rate constant k_2 (Figure 5). The variations with tubulin concentration observed in the experimental curves of GTP hydrolysis are much less important than the variations of the turbidity curves and are due

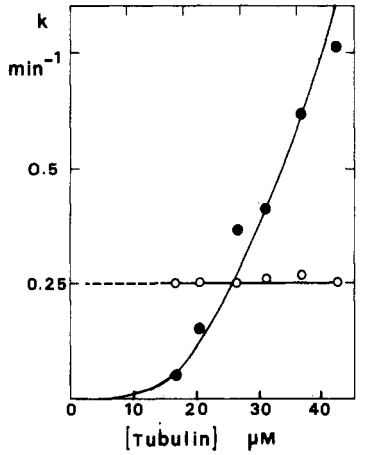

FIGURE 5: Plot of the rate constants for tubulin polymerization, k_1, and for GTP hydrolysis by polymerized tubulin, k_2, vs. tubulin concentration. Values of k_1 (●) were obtained here from analysis of the turbidity curves, and values of k_2 (○) were from the computer adjustment to eq 1 of the experimental data shown in Figure 3.

uniquely to the dependence of k_1 on concentration, in eq 1.

The evolution of turbidity with time obeys the equation

$$[\text{MT}] = [\text{T–GDP}]_{\text{MT}} + [\text{T–GTP}]_{\text{MT}} = 1 - e^{-k_1 t}$$

Consequently, the difference at each time between the normalized values of the amount of microtubules and of P_i produced represents the proportion of the transient tubulin–GTP complex in the microtubule, the evolution of which is described by the equation

$$[\text{T–GTP}]_{\text{MT}}(t) = \frac{k_1}{k_1 - k_2}(e^{-k_2 t} - e^{-k_1 t}) \quad (2)$$

The higher the tubulin concentration, the larger the difference between k_1 and k_2, and the higher the maximum amount of T–GTP complex in the microbutules, reached at a time $t_{\text{GTPmax}} = [1/(k_1 - k_2)] \log (k_1/k_2)$.

(2) Exchangeability of GTP in the Microtubules. When the steady-state rate of GTP hydrolysis has been reached, only GDP is bound to polymerized tubulin at the E site and is known to be nonexchangeable (Jacobs et al., 1974; Kobayashi, 1975; Weisenberg et al., 1976; David-Pfeuty et al., 1977). The question arises from the above results whether GTP is exchangeable in the $[\text{tubulin–GTP}]_{\text{microtubule}}$ complex. In order to answer this question, the following experiment was devised: tubulin at a concentration of 30 μM was polymerized in the presence of 180 μM [γ-^{32}P]GTP, and the time courses of both the extent of assembly and GTPase activity were followed. In a parallel sample, a 2 mM unlabeled GTP chase was performed at time 3 min, when 90% of the microtubules were formed and only 30% of GTP had been hydrolyzed on the polymerized tubulin. If GTP bound to polymerized tubulin is unexchangeable, the hydrolysis of the 70% [γ-^{32}P]GTP remaining on the microtubules and the liberation of $^{32}P_i$ at its initial specific radioactivity are expected to continue after the isotopic dilution (Figure 6, dashed line). On the contrary, if GTP bound to microtubules is rapidly exchangeable, the chase of [γ-^{32}P]GTP should take place at the E site in the microtubule as well as on the free tubulin, and the subsequent liberation of $^{32}P_i$ should take place with an 11-fold lower specific radioactivity. Figure 6 shows that this latter situation is indeed what happens. The final diluted specific radioactivity of the liberated $^{32}P_i$ is established after 45 s, and the observed radioactivity burst after the chase represents only 17% of the amplitude of the burst which would be expected in the case of nonexchangeability of bound GTP; in the case of total

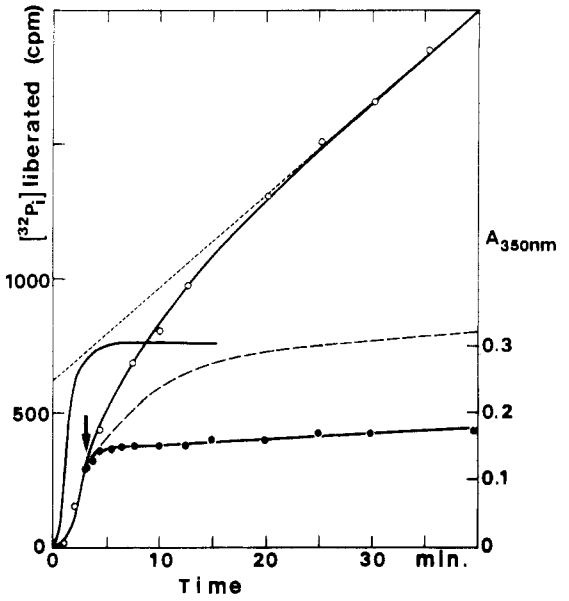

FIGURE 6: Effect of unlabeled GTP chase on GTP hydrolysis during tubulin polymerization. Tubulin at a concentration of 30 μM was polymerized in P buffer containing 181 μM [γ-^{32}P]GTP. The specific radioactivity of $^{32}P_i$ in the assay was 21.5 cpm/μM. (Full line) Recorded turbidity; (O) $^{32}P_i$ liberated during polymerization. In a parallel assay, at the time indicated by the arrow, when 91% of the total amount of microtubules was formed, an isotopic dilution with 2 mM unlabeled GTP was performed, and subsequent $^{32}P_i$ liberation was measured (●). (Dashed line) Calculated theoretical evolution of $^{32}P_i$ after the isotopic dilution, assuming the hypothesis that [γ-^{32}P]GTP bound to microtubules is not exchangeable with the unlabeled GTP added.

instantaneous exchangeability, the 11-fold isotopic dilution would yield an apparent burst of 9% of the original [γ-^{32}P]-GTP bound to microtubules. The number obtained is then very close to this latter value. This result suggests that at 37 °C the exchange of GTP on polymerized tubulin is fast, and the rate of exchange is of the same order as on the unpolymerized tubulin dimer (Huitorel, 1979).

The exchangeability of GTP bound to polymerized tubulin was further checked in another experiment: tubulin at a concentration of 28 μM was polymerized in the presence of 150 μM of either ^{3}H- or γ-^{32}P-labeled GTP. The evolution of several parameters was followed during the time course of assembly: (1) turbidity, (2) GTP hydrolysis, (3) the weight of microtubules, and (4) [^{3}H]GDP incorporation. Aliquots (125 μL) of the tubulin solution were polymerized with 150 μM [^{3}H]GTP in the rotor of the airfuge maintained at 37 °C. At different time intervals, an unlabeled GTP chase was performed by adding 1 μL of 200 mM GTP solution in the respective tubes, and this was followed by immediate centrifugation. In a separate tube, contamination of the pellets was estimated by adding both tracing [^{3}H]GTP and the unlabeled GTP chase at the same time immediately prior to centrifugation. The extent of polymerization at each time was determined from the protein content of the supernatant in each tube, and the amount of ^{3}H-labeled nucleotide contained in the pellets gave the concentration of unexchangeable [^{3}H]GDP remaining on the microtubules after chasing exchangeable [^{3}H]GTP with unlabeled GTP. Figure 7 shows the pooled data. At each time of the assembly process, a satisfactory superposition was obtained for the corrected turbidity and the weight of microtubules, on the one hand, and for the amount of P_i produced and the number of [^{3}H]GDP measured per molecule of tubulin incorporated in the microtubule, on the other hand. The difference, at each time, between these two curves represents the evolution with time of the transient complex $[T\text{–}GTP]_{microtubules}$. The theoretical evolution with

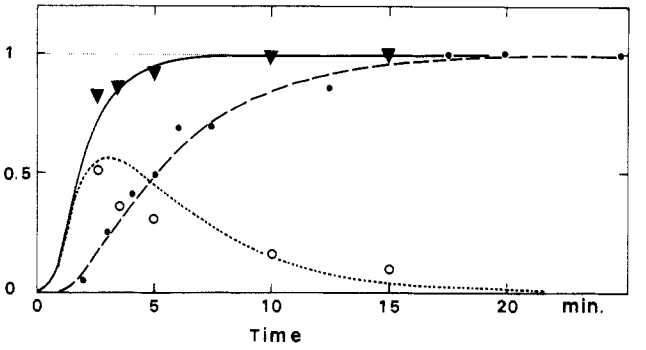

FIGURE 7: Correlation between different parameters in the time course of tubulin polymerization. Tubulin, at a concentration of 30 μM, was polymerized in the presence of 178 μM GTP, either γ-^{32}P-labeled for GTP hydrolysis measurement or ^{3}H-labeled for determination of GDP content of microtubules, at different times as described under Materials and Methods. (Full line) Recorded turbidity; (▼) amount of microtubules, determined by rapid high-speed centrifugation; (●) burst of P_i production; (O) amount of ^{3}H-labeled nucleotide displaced from polymerized tubulin E site after a 2 mM unlabeled GTP chase (this value is normalized with respect to the final amount of polymerized tubulin of 18.6 μM measured at time 15 min). (Dashed line and dotted line) Theoretical curves for the evolutions of the polymerized tubulin–GDP and transient tubulin–GTP complexes, respectively, calculated by using eq 1 and 2, and the numerical values $k_1 = 0.69$ min^{-1} and $k_2 = 0.24$ min^{-1}.

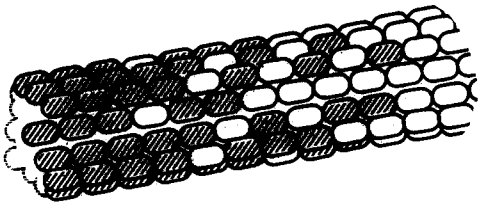

FIGURE 8: Model for an elongating microtubule in the presence of GTP, and featuring the gradient of T–GTP along the growing microtubule. The darkened segments represent the tubulin–GTP complex while the unmarked segments represent the tubulin–GDP complex.

time of the polymerized T–GDP and T–GTP complexes predicted by eq 1 and 2 has been simulated on Figure 7 by using the numerical values $k_1 = 0.7$ min^{-1} and $k_2 = 0.25$ min^{-1} and shows a reasonable agreement with the experimental data.

Discussion

The results presented show that GTP is hydrolyzed at the exchangeable site of tubulin after tubulin assembly. Although polymerization and GTP hydrolysis proceed in a compulsory order, there is no tight kinetic coupling between the two events, since the GTPase activity is a kinetic process characteristic of polymerized tubulin and takes place as a first-order reaction whose rate constant is independent of the initial tubulin concentration, contrary to the polymerization reaction. A consequence of these data is the existence of a time-dependent gradient of the ratio ([GTP]/[GDP])(t) along the microtubules, the end which contains newly polymerized tubulin being richer in GTP than the previously assembled body of the microtubule (Figure 8). This model is different from the one presented by Karr et al. (1979) in which there is a GTP boundary at the end of the microtubule only, which would represent at most 1% of the total nucleotide bound to the microbutule. This latter model cannot account for the data, except perhaps at steady state, and would best agree with the case of tight kinetic coupling between polymerization and GTP hydrolysis (our first model) which has been rejected by the experimental evidence. When tubulin is assembled at high concentration, polymerization proceeds very fast ($k_1 \sim 1$ min^{-1}), and a maximum molar fraction of ~0.7 GTP per polymerized tubulin molecule can be reached at time $t_{GTPmax} \simeq 2$ min. This indicates that microtubules carrying GTP do

exist, and if their relative stability compared with that of GDP–microtubules is not known from these experiments, however, their existence corroborates the reported possibility of obtaining microtubules in the presence of nonhydrolyzable analogues of GTP. However, it has not been possible to obtain stable GTP–microtubules without subsequent hydrolysis of GTP. For instance, highly stable microtubules can be obtained in the presence of taxol (Schiff et al., 1979), and we checked that even at 0 °C microtubules could be obtained in the presence of micromolar concentrations of taxol under our assembly conditions; however, in this case also, hydrolysis of GTP accompanies tubulin·polymerization (data not shown). On the other hand, it is interesting to note that in polymerization experiments done in the presence of Cr^{III}GTP MacNeal & Purich (1978b) observed the hydrolysis of only a fraction of this analogue on the microtubules after 30 min of polymerization, which indicates that in this system GTP hydrolysis could be slowed down.

Quite recently, Weisenberg (1980) proposed a theoretical model for microtubule assembly, in which the possible relationships between polymerization and GTP hydrolysis are emphasized. One interest of this model comes from the idea of different rates of GTP hydrolysis of tubulin during assembly whether tubulin is laterally interacting with one or two tubulin protomers in the microtubule wall. Our results show that GTP is hydrolyzed one time only at a slow rate and does not turnover when tubulin is sequestered in the wall and interacting with four subunits. On the other hand, if we assume that the linear GTP hydrolysis at steady state is due to boundary tubulin located at microtubule ends, and interacting with two or three subunits only, it appears that in this case there is an actual turnover of GTP hydrolysis. Since a very small proportion of tubulin is located at the ends of microtubules, the observed steady-state rate corresponds to a much higher rate constant than in the body of the microtubules. Further experiments, however, are needed to clarify the quantitative relationships between GTP hydrolysis and tubulin–tubulin interactions.

During microtubule steady state, the body of microtubules contains GDP only, and GTP hydrolysis proceeds essentially at microtubule ends. This irreversible reaction is the support for the "treadmilling" model proposed by Margolis & Wilson (1978). This model implies that the two ends of microtubules are in some way different, namely, a polymerizing end and a dissociating end. Moreover, structural studies indicate that tubulin $\alpha\beta$ dimers are arranged in the microtubule in a polarized way, giving a β end and an α end. Since the GTP E site is on the β subunit, it may be that GTP is hydrolyzed almost only at the β end, the β subunit being blocked inside the microtubule at the other end. In this model, the β end would be the polymerizing end, and the α end the dissociating end.

It must be pointed out that very similar features characterizing the involvement of nucleotide triphosphate hydrolysis in polymer formation and the steady state are exhibited by the actin system (Cooke & Murdoch, 1973; Brenner & Korn, 1980a,b). Although quantitative kinetic studies of this connection are not available in this system, a theoretical work predicted that assembly and hydrolysis should be uncoupled (Cooke, 19755), and recent preliminary data support this hypothesis (Pardee & Spudich, 1979).

Our results are not conflicting with the data of MacNeal & Purich (1978a), who studied the tubulin polymerization and concomitant GTP hydrolysis of a preformed tubulin–[γ-^{32}P]GTP (1:1) complex and found that both events "proceeded at nearly identical rates". Indeed, a slight delay between the two events could be observed in their data, which later brought the authors themselves to suspect a possible uncoupling between the two processes.

Our kinetic data confirm that also in the presence of free GTP in the medium a burst of one P_i molecule is produced per tubulin dimer incorporated in the microtubule. This evidence corroborates the previous finding that there is no turnover of the nucleotide at the E site on the microtubules: as soon as GTP is hydrolyzed, GDP is not exchangeable on the microtubule. However, when the tubulin material was purified through phosphocellulose instead of DEAE-Sephadex chromatography, a burst of 1.5–1.8 P_i per polymerized tubulin was often obtained. The reason for this higher value remains obscure. It could be due to a slight turnover of GTP preceding the blockage of the E site on the microtubule or to the presence of some copurified protein which would have a global GTPase activity related in some way to the polymerization: a nucleoside diphosphokinase activity, for instance, has been found associated to phosphocellulose-treated tubulin, and not to DEAE-Sephadex-treated tubulin (Jacobs & Huitorel, 1979).

The fact that GTP bound to polymerized tubulin is exchangeable while GDP is not suggests, as a working hypothesis, the involvement of a conformation change in the assembly process. This conformational change of tubulin following assembly either could trigger GTP hydrolysis, which would result in the blockage of GDP in the E site, the tubulin–GDP complex having a more stable conformation than the tubulin–GTP complex, or could take place after GTP hydrolysis, and utilize the energy liberated by the splitting of GTP to stabilize the interaction between the molecules of tubulin–GDP. This question presents some analogies with the theories proposed in the case of phosphorylations coupled to energetic phenomena (Boyer et al., 1973) in which energy is driven through conformational changes of proteins. Some support to a different conformation of the nucleotide site when GTP or GDP is bound is brought by fluorescence (Karr & Purich, 1978) and photoaffinity labeling data (Geahlen & Haley, 1979). Some insight in the hypothetical conformation change of tubulin following assembly could be brought with the use of analogues of GTP fluorescent probes (Wiegand & Kaleja, 1976). This problem and the related point of the possible different stability of GTP– and GDP–microtubules are currently under examination in this laboratory.

References

Arai, T., & Kaziro, Y. (1976) *Biochem. Biophys. Res. Commun. 69*, 369–376.

Avron, M. (1960) *Biochim. Biophys. Acta 40*, 257.

Boyer, P. D., Cross, R. L., & Momsen, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2837.

Brenner, S. L., & Korn, E. D. (1980a) *J. Biol. Chem. 255*, 841–844.

Brenner, S. L., & Korn, E. D. (1980b) *J. Biol. Chem.* (in press).

Carlier, M.-F., & Pantaloni, D. (1978) *Biochemistry 17*, 1908–1915.

Carlier, M.-F., & Pantaloni, D. (1980a) *International Congress of Cell Biology, 2nd*, Berlin, West Germany.

Carlier, M.-F., & Pantaloni, D. (1980b) *International Symposium on Microtubules and Microtubule Inhibitors*, Beerse, Belgium.

Chien, J.-Y. (1948) *J. Am. Chem. Soc. 70*, 2256–2261.

Cooke, R. (1975) *Biochemistry 14*, 3252–3256.

Cooke, R., & Murdoch, L. (1973) *Biochemistry 12*, 3927–3932.

David-Pfeuty, T., Erickson, H. P., & Pantaloni, D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5372–5376.
David-Pfeuty, T., Simon, C., & Pantaloni, D. (1979) *J. Biol. Chem. 254*, 11696–11702.
Geahlen, R. L., & Haley, B. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4375–4377.
Geahlen, R. L., & Haley, B. E. (1979) *J. Biol. Chem. 254*, 11982–11987.
Huitorel, P. (1979) M.S. Thesis, Orsay.
Jacobs, M., & Huitorel, P. (1979) *Eur. J. Biochem. 99*, 613–622.
Jacobs, M., Smith, H., & Taylor, E. W. (1974) *J. Mol. Biol. 89*, 455–468.
Karr, T. L., & Purich, D. L. (1978) *Biochem. Biophys. Res. Commun. 84*, 957–961.
Karr, T. L., Podrasky, A. E., & Purich, D. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5475–5479.
Kobayashi, T. (1975) *J. Biochem. (Tokyo) 77*, 1193–1197.
Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183–5187.
Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.
MacNeal, R. K., & Purich, D. L. (1978a) *J. Biol. Chem. 253*, 4683–4687.
MacNeal, R. L., & Purich, D. L. (1978b) *Arch. Biochem. Biophys. 191*, 233–243.
Margolis, R. L., & Wilson, L. (1978) *Cell (Cambridge, Mass). 13*, 1–8.
Murphy, D. B., Vallee, R. B., & Borisy, G. G. (1977) *Biochemistry 16*, 2598–2605.
Pardee, J., & Spudich, J. A. (1979) *Cold Spring Harbor Symp., Abstr.*, 27.
Penningroth, S. M., & Kirshner, M. W. (1977) *J. Mol. Biol. 115*, 643–673.
Schiff, P. B., Fant, J., & Hortwitz, S. B. (1979) *Nature (London) 277*, 665–667.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.
Wegner, A. (1976) *J. Mol. Biol. 108*, 139–150.
Weisenberg, R. C. (1980) *J. Mol. Biol. 139*, 660–677.
Weisenberg, R. C., & Deery, W. J. (1976) *Nature (London) 263*, 792.
Weisenberg, R. C., Deery, W. J., & Dickinson, P. J. (1976) *Biochemistry 15*, 4248–4254.
Wiegand, G., & Kaleja, R. (1976) *Eur. J. Biochem. 65*, 473–479.
Zackroff, R. V., Weisenberg, R. C., & Deery, W. J. (1980) *J. Mol. Biol. 139*, 641–677.

Conformation and Assembly Characteristics of Tubulin and Microtubule Protein from Bovine Brain†

David C. Clark, Stephen R. Martin, and Peter M. Bayley*

ABSTRACT: The conformational requirements for the efficient assembly of bovine brain tubulin into microtubules have been investigated by using near-UV circular dichroism. Microtubule protein was prepared by the assembly–disassembly method of Shelanski et al. [Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768]. Tubulin dimer, isolated from this multiprotein complex by phosphocellulose ion-exchange chromatography in the presence and absence of Mg^{2+}, was compared with tubulin dimer (WT dimer) prepared by the method of Weisenberg & Timasheff [Weisenberg, R. C., & Timasheff, S. N. (1970) *Biochemistry 9*, 4110–4116]. The tubulin from both dimer preparations showed identical electrophoretic patterns in which high molecular weight protein was undetectable. However, reproducible and significant differences were found in the near-UV CD spectra. Phosphocellulose-treated tubulin resembles the original microtubule protein more closely than does WT dimer, although this latter material has been widely accepted as being representative of the native protein. The phosphocellulose-treated tubulin and WT dimer are not readily interconvertible by simple physical or chemical treatments. The assembly capability of the various tubulin dimer preparations was compared by measuring the enhancement by tubulin dimer of assembly of ring fraction (isolated from microtubule protein by gel filtration on Sepharose 6B). Again phosphocellulose-treated tubulin is found to behave more like native microtubule protein than does WT dimer.

Microtubules, organelles found in all eukaryotic cells, have been the subject of considerable interest in recent years and reviewed recently (Snyder & McIntosh, 1976; Jacobs & Cavalier-Smith, 1977; Kirschner, 1978; Dustin, 1978). Some of the cellular functions which involve microtubules (e.g., mitosis) are dependent upon the ordered assembly and disassembly of these structures. Also, the binding of certain alkaloid drugs (e.g., colchicine; Wilson et al., 1974) and the variation in a number of external physical parameters (e.g., low temperature and high pressure) have been shown to dissociate microtubules reversibly in eukaryotic cells, leading to disruption of a variety of cellular functions (Olmsted & Borisy, 1973) and providing evidence for the involvement of microtubules in chromosome movements in cell division, intracellular organization and transport, development and maintenance of cell shape, cellular motility, and sensory transduction.

A number of different methods of isolation have been developed for the proteins comprising microtubules, including the essentially classical biochemical isolation via ammonium sulfate precipitation and column chromatography to yield

† From the Biophysics Division, National Institute for Medical Research, Mill Hill, London, NW7 1AA England. *Received August 5, 1980.*

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