cellular pH; this is exactly what is observed in NR70 cells. ^{31}P NMR results demonstrate that in NR71 cells, the rate at which the NTP pool disappears in the absence of an exogenous carbon source is less than that in the NR70 cells (Figure 9); the specific reactions responsible for this are not known. Furthermore, NR71 cells accumulate large amounts of a phosphodiester compound (resonance X in Figure 9a) which has not been identified. Clearly, there exist some unexplained differences between the intermediary metabolism of strains NR70 and NR71; these differences could have arisen as an indirect consequence of the higher pH^{in} levels maintained by the NR71 cells.

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Mechanism of Tubulin Assembly: Guanosine 5′-Triphosphate Hydrolysis Decreases the Rate of Microtubule Depolymerization†

Dominique Bonne* and Dominique Pantaloni

ABSTRACT: The rate of depolymerization of microtubules upon lowering the temperature was found to depend on the amount of time elapsed since the beginning of the assembly process. In the first minutes following self-assembly at 37 °C, microtubules are more cold sensitive and depolymerize faster than later at the steady state. In the meanwhile, no change occurred in the average length nor in the shape of the distribution of microtubules. On the other hand, the evolution with time of the apparent dissociation rate constant of tubulin from microtubules was in good correlation with the GTP content of microtubules following assembly, showing that GTP hydrolysis modifies the tubulin–tubulin interactions. Microtubule-bound GTP was not exchangeable for GDP, but steady-state GTP hydrolysis was inhibited by GDP. This result indicates that GDP and GTP exhibit different affinities for tubulin in the body and at the ends of microtubules. It is proposed that GTP–tubulin dissociates faster from microtubules than GDP–tubulin. In other words GTP hydrolysis contributes to the stabilization of microtubules.

Since the discovery of a guanyl nucleotide requirement for microtubule polymerization (Weisenberg, 1972), and GTP hydrolysis during polymerization (Berry & Shelanski, 1972), the exact role of nucleotide hydrolysis in the structure and function of microtubules remains unknown.

It is well established that GTP hydrolysis occurs 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).

The stoichiometry is one GTP hydrolyzed per molecule of tubulin dimer incorporated in the polymer (McNeal & Purich, 1978).

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Nevertheless, GDP allows the elongation of microtubules if not nucleation (Carlier & Pantaloni, 1978; Karr et al., 1979; Zackroff et al., 1980). Moreover, microtubules can be obtained in the presence of large amounts of nonhydrolyzable analogues of GTP (Arai & Kaziro, 1976; Penningroth & Kirschner, 1977; Weisenberg & Deery, 1976; Terry & Purich, 1980) and appear less sensitive to cold or Ca^{2+}-induced depolymerization than microtubules containing GDP at the E site. So it was suggested that the role of GTP hydrolysis during microtubule assembly was to allow further depolymerization and that this was important in regulation of microtubule–tubulin equilibrium in vivo.

Recent work from this laboratory (Carlier & Pantaloni, 1981) demonstrated that GTP hydrolysis was not strictly coupled to polymerization and occurred in a subsequent step. Consequently, in the early stage of assembly, a GTP–tubulin complex is the transient major constituent of microtubules.

It was found relevant to inquire about differential properties of microtubules in relation with their nucleotide content. The property studied here is the cold sensitivity of microtubules. Microtubules polymerized at 37 °C were disassembled at 21 °C, a temperature at which the depolymerization process was slow enough to allow the accurate measurement of the apparent dissociation rate constants.

We show that at 21 °C the GTP–tubulin complex dissociates from microtubules with a larger apparent rate constant than GDP–tubulin. So, at variance with previous results (Penningroth & Kirschner, 1977; Weisenberg & Deery, 1976), it appears that GTP hydrolysis does not promote a destabilization of microtubules but, on the contrary, decreases tubulin ability to dissociate from microtubules.

Materials and Methods

Reagents. 2-(*N*-Morpholino)ethanesulfonic acid (Mes)[1] was purchased from Calbiochem, guanosine 5′-triphosphate trilithium salt from Boehringer, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) from Sigma Chemical Co., and DEAE-Sephadex A-50 from Pharmacia; glycerol and all salts used in buffers were Merck's analytical grade. Guanosine 5′-[γ-^{32}P]triphosphate was purchased from Amersham.

Tubulin Purification. Tubulin was purified from fresh pig brain by three consecutive cycles of assembly–disassembly, according to Shelanski et al. (1973), followed by gel chromatography through DEAE-Sephadex A-50 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 following 2–3 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). Alternatively, purification of tubulin was carried out on phosphocellulose according to the procedure of Weingarten et al. (1975).

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 with a Beckman Acta V recording spectrophotometer equipped with a thermostated 100-μL and 0.5-cm light path cell connected with a T-jump apparatus. The polymerization reaction was started by a jump in temperature from 4 to 37 °C. At any time, the temperature can be turned off to 21 or 4 °C by a direct change of circulating water. The half-times for these temperature jumps have been measured in the same cell by the change in absorbance at 558 nm of a phenol red solution in Tris buffer, pH 8 ($\Delta pH/\Delta T$ = 0.03 pH unit/deg), and are respectively 15, 8, 10, and 10 s for the following temperature jumps: 4 to 37 °C, 37 to 21 °C, 37 to 4 °C, and 21 to 4 °C. The observed turbidity values at 350 nm were corrected as described (Carlier & Pantaloni, 1978) in order to obtain data directly proportional to the amount of polymer formed.

Direct Measurement of Microtubule Weight. 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 by using a prewarmed rotor at 37 °C and a warm air circulator which maintained the rotor temperature at least at 30 °C in order to avoid depolymerization of microtubules during the centrifugation.

In the determination of microtubule weight, a correction was made for the occasional formation of aggregates by cooling at 4 °C and centrifuging parallel samples at 20 °C for 2 min. The amount of microtubules was then calculated from the difference between the concentrations of the supernatants of the cold and warm centrifugation. This correction for aggregate formation, although always weak, was not negligible.

The measurement of the concentrations of free and exchangeable GTP in the assay is important for the accurate determination of labeled GTP specific radioactivities in the measurement of [γ-^{32}P]GTP hydrolysis.

The concentration of free and bound (exchangeable and nonexchangeable) guanine nucleotides present in tubulin solutions was determined spectrophotometrically after 4% perchloric acid extraction by 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 a proportion of 1:1 to tubulin.

GTPase Activity Measurements. The GTPase activity of tubulin during polymerization was monitored by the extraction 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 50-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 accomplished by use of 2-methyl-1-propanol–cyclohexane–acetone–ammonium molybdate (750:750:15:1) solution saturated with water. A 1-mL aliquot of the organic phase was counted in 10 mL of Aquasol.

[1] Abbreviations: E site, exchangeable binding site on tubulin; GMP-PCP, guanosine 5′-(β,γ-methylenetriphosphate); GMP-PNP, guanosine 5′-(β,γ-imidotriphosphate); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

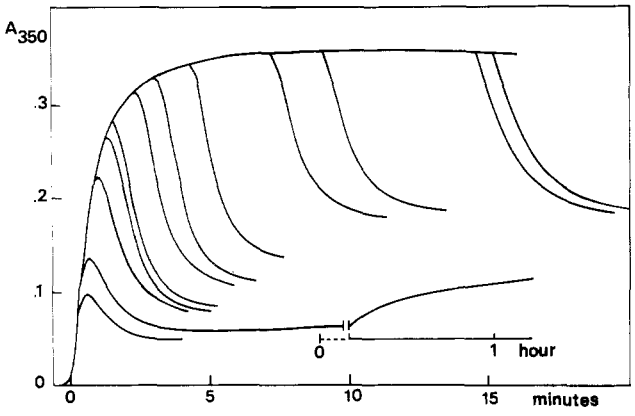

FIGURE 1: Time course of 21 °C depolymerization of 37 °C assembled microtubules at different times after the beginning of polymerization. Tubulin, at a concentration of 35 μM, was polymerized at 37 °C in buffer P containing 250 μM GTP.

Electron Microscopy Analysis. Microtubules samples were negatively stained with 2% uranyl acetate on 7.5-mesh grids after 0.2% isothermal glutaraldehyde fixation followed by 1:20 dilution.

Microtubule Length Distribution. Electron photomicrographs at a final 2000–2500-fold magnification were used to determine the length distribution histograms. A total of 600–1000 microtubules were measured. Processing of the data was performed by a Wang 720 computer, giving the histogram of the length distribution and the mean length of the distribution. The theoretical depolymerization curve was calculated from the histogram by assuming a linear endwise depolymerization for each microtubule (Johnson & Borisy, 1977).

Results

Evolution of the Rate of Depolymerization at 21 °C with the Age of Microtubules. Tubulin assembly was monitored by turbidity at 37 °C in buffer P containing 250–1000 μM GTP and 15–40 μM tubulin. At different times after the beginning of assembly, a temperature jump from 37 to 21 °C was performed and microtubule depolymerization was followed as shown in Figure 1. As soon as thermal equilibrium was attained, the kinetics of disassembly mainly consisted in an exponential decay. After transformation of the turbidity data (A_{350}) into the weight concentration of microtubules, c_w, the curves were fitted to a first-order process $c_w = ae^{-kt}$ where k is the first-order rate constant of depolymerization at 21 °C. The constant k actually represents the intrinsic subunit dissociation rate constant k' corrected by a factor reflecting the length distribution of microtubules which depends on tubulin concentration. Figure 1 shows that the time course of disassembly appeared different according to the time at which the temperature was switched from 37 to 21 °C during the polymerization process. The extent and the initial rate of depolymerization both reached a maximum about 3 min after the beginning of assembly, the time at which 95% microtubules were formed. Later, when the turbidity plateau was established, the initial rate of disassembly decreased and reached a stable minimum when microtubules where older than 15 min. It was repeatedly observed that this rate of depolymerization remained constant when disassembly was started at times as long as 60 min after the beginning of assembly. In Figure 1, in which the experiment was conducted with 35 μM tubulin, $k_-{}^{21}{}_{cbsd}$ values varied from 1.4 min^{-1} to 0.9 min^{-1} for respectively 3- and 15-min-old microtubules.

When disassembly at 21 °C was started in the early times of the polymerization process, an undershoot in turbidity was observed in the depolymerization time course, a low level of turbidity A_m being followed by a slow repolymerization process up to the final level A_f. The value of the undershoot $A_f - A_m$ was a decreasing function of the age of the microtubules, the final level of turbidity A_f being reached straight off when the depolymerization at 21 °C was started at the late times of the assembly process at 37 °C. The same equilibrium level could be reached, yet at a slow rate, by polymerizing the tubulin solution from 4 to 21 °C.

The observed time dependence of $k_-{}^{21}{}_{obsd}$ was not due to an accumulation of reaction products. The same phenomenon was repeatedly observed during another polymerization cycle following disassembly as long as the experiment was conducted in the presence of enough GTP.

It can be noted that the same behavior was observed for microtubules polymerized from a cycled tubulin preparation, containing associated proteins, in a glycerol-free polymerizing medium.

Length Distribution of Microtubules following Assembly. If it is assumed that microtubules depolymerize by endwise subunit dissociation (Oosawa & Asakura, 1975; Johnson & Borisy, 1977; Karr et al., 1980), the initial rate of disassembly, after dilution or cold treatment, is proportional to the number concentration of microtubule ends. The observed change in the rate of cold disassembly with the age of microtubules could reflect a decrease in the number concentration of microtubule ends during the assembly process. Such a behavior would be contradictory to the Oosawa & Asakura (1975) theory for nucleated polymerization. Previous kinetic studies of microtubule formation under the same conditions (Carlier & Pantaloni, 1978) 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 assembly. A variation in microtubule number concentration could only be due to a redistribution in microtubule length following assembly.

In order to check this hypothesis, we measured the length distribution of microtubules from negative-staining electron micrographs performed at a short (3 min) and a long (26 min) time after the beginning of assembly at 37 °C. Histograms of a length distribution of 700–900 microtubules (Figure 2) were found to fit a Poisson distribution rather than an exponential or spike one. In three separate determinations, no major difference was detectable between histograms performed at different times. Typically, at a tubulin concentration of 30 μM, average lengths of 4.95 and 5.0 μM were measured at time 3 and 26 min, respectively.

From each experimental histogram, theoretical depolymerization curves were calculated within the endwise dissociation model (Figure 2, inset) according to Johnson & Borisy (1977) and were found mostly identical. The experimental apparent rate constants of depolymerization at 21 °C, expressed in min^{-1}, were compared with the corresponding theoretical rate constants expressed in dimers per arbitrary time unit. This unit is the time necessary for each microtubule to shorten 0.714 μm, i.e., for the shortest microtubule class to disappear. From this comparison, the intrinsic rate constants of depolymerization at 21 °C could be calculated. Values of 220 and 140 dimers/s were found for 3- and 26-min-old microtubules, respectively. Anyway, the observed variation of $k_-{}^{21}$ is not due to a length redistribution of microtubules involving a decrease in the number of depolymerizing species throughout the assembly cycle.

Oosawa & Asakura's (1975) theory predicts a redistribution of polymer sizes which would turn from the Poisson distri-

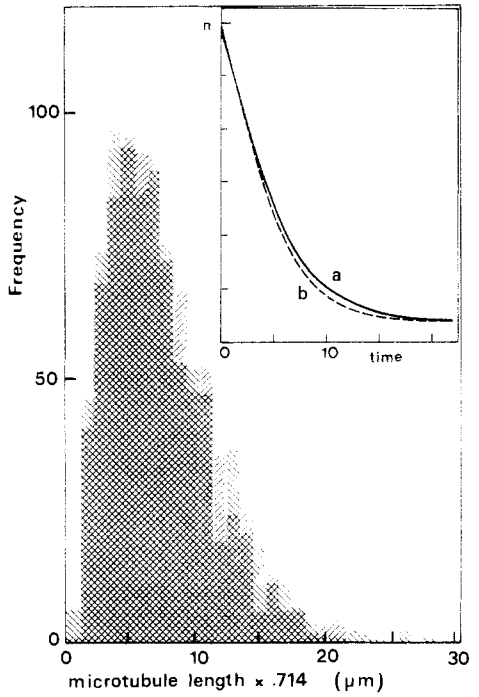

FIGURE 2: Length distribution of microtubules (tubulin = 30 μM) at two different times after 37 °C assembly (length classes of 714 nm). (▨) 3 min (621 counted microtubules); (▩) 26 min (670 counted microtubules). (Inset) Theoretical depolymerization curves calculated from the corresponding histograms: (a) 3 min; (b) 26 min. Time is expressed with arbitrary units.

bution to an exponential type with a time of approach to the final equilibrium of τ_2. This time, τ_2 depends on the mean length of microtubules (i) and the off rate constant k_- according to

$$\tau_2 = i^2/(2k_-)$$

In our experiments the value of τ_2 would be about 20 h. In agreement with this calculation, during the time of our experiments (1 h), we did not detect any change in the length distribution of microtubules which mainly obeyed the Poisson law (Figure 2). Therefore, the hypothesis of a length redistribution is ruled out in this case.

Correlation of the Apparent Rate Constant of 21 °C Depolymerization with the GTP Content of Microtubules. It has been demonstrated previously (Carlier & Pantaloni, 1981) that GTP hydrolysis is not a limiting step in tubulin polymerization and takes place as a subsequent uncoupled first-order process. Comparison of the two kinetic processes (Figure 3) revealed that essentially GTP is bound to microtubules during the first minutes of assembly. Figure 3 clearly shows that the change in the disassembly rate constant at 21 °C follows the same evolution as the GTP content of microtubules, derived from the simultaneous measurement of GTP hydrolysis during assembly, both being maximum at the same time (2–3 min of assembly at the observed tubulin concentration of 35 μM). At time 15 min of the assembly process, the GTP content of microtubule became very low and the observed k_-^{21} reached a stable minimum value.

A linear correlation (Figure 4) was established between the GTP content of microtubules (expressed as the ratio of moles of GTP per mole of incorporated tubulin) and the observed disassembly rate constant $k_-^{21}{}_{obsd}$ at 21 °C for depolymerizations performed at times longer than 2 min after the beginning of assembly. Extrapolations of this plot to 0 and 1 mol of GTP/mol of incorporated tubulin lead to the apparent disassembly rate constants at 21 °C for microtubules carrying

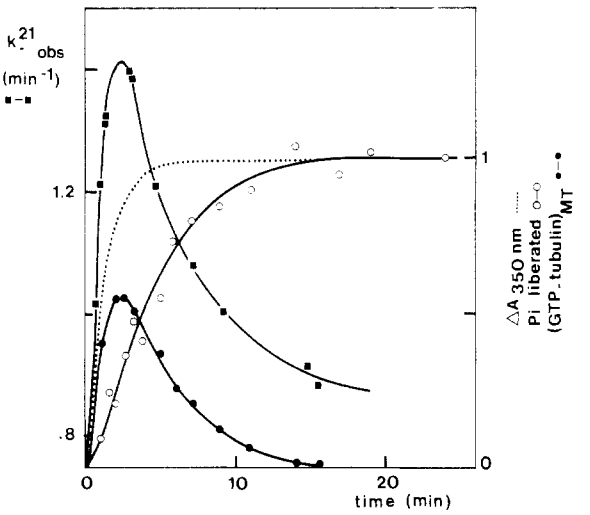

FIGURE 3: Variation of the apparent rate constant of depolymerization at 21 °C as a function of time after the beginning of assembly. Concomitant time course of formation of microtubules, accompanying GTP hydrolysis and GTP content of microtubules. (■) Apparent off rate constant ($k_-^{21}{}_{obsd}$); (···) turbidity data, normalized with respect to the final amount of microtubules; (○) burst of P_i production during assembly obtained after subtraction of the linear steady-state GTP hydrolysis from the whole phenomenon; (●) transient GTP–tubulin incorporated in the microtubule defined as the difference, at each time, between the total amount of polymerized tubulin (turbidity data) and the amount of GDP bound to microtubules (burst of P_i accompanying assembly).

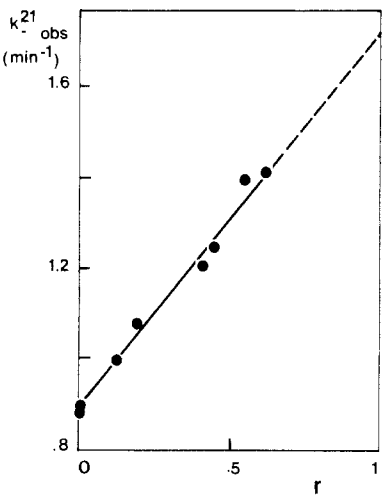

FIGURE 4: Correlation between the apparent off rate constant at 21 °C, $k_-^{21}{}_{obsd}$, and r, the proportion of E sites in polymerized tubulin occupied by GTP (expressed in moles of incorporated GTP per mole of polymerized tubulin). Experimental conditions are those of Figure 1.

only GDP or only GTP, respectively. In the conditions of the shown experiment, the $k_-^{21}{}_{obsd}$ for polymerized GTP–tubulin appears to be 2 times larger than $k_-^{21}{}_{obsd}$ for polymerized GDP–tubulin. When disassembly at 21 °C was performed during the elongation process, i.e., at times shorter than 3 min, the disassembly rate constants were lower than expected for microtubules carrying essentially GTP. This deviation from the expected law was most probably due to the large proportion of very short microtubules present at these early times of assembly. The early total disassembly of these short microtubules results in a decrease in the average number of microtubules present during the major part of the disassembly process.

It has been shown that at low tubulin concentration, the 37 °C polymerization process is slow and GTP hydrolysis is

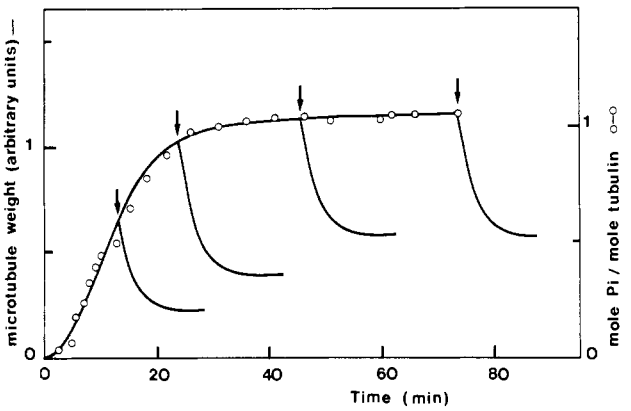

FIGURE 5: Depolymerization curves at 21 °C of microtubules assembled at 37 °C at low tubulin concentration (1 mg/mL). (—) Evolution of the microtubule weight from the turbidity data corrected as indicated in the text; (O) burst of P_i production during assembly obtained after subtracting the steady-state GTP hydrolysis to the total activity. At times 12, 24, 44, and 74 min, disassembly at 21 °C proceeded with the following dissociation rate constants: 0.38, 0.39, 0.36, and 0.38 min^{-1}, respectively.

Table I: Apparent Rate Constants vs. Tubulin Concentration

tubulin concn (mg/mL)	k_-^{21}GDP (min^{-1})	k_+^{37} (min^{-1})	k_-^{21}GDP/k_+^{37}
0.75	0.125	0.055	2.27
1	0.39	0.17	2.29
1.25	0.68	0.32	2.12
1.5	1.01	0.474	2.13
2	1.35	0.59	2.28
2.5	1.48	0.64	2.30
3	1.61	0.70	2.30
		av:	2.24 ± 0.08

strictly coupled to microtubule assembly. In this case, GDP only is present on the microtubules all along the polymerization curve (Figure 5), and therefore a unique value of the apparent depolymerization rate constant k_-^{21} should be obtained. Figure 5 shows that the disassembly rates measured at different times of the assembly process were identical, as expected within the proposed model.

Dependence of the Rate of Depolymerization of Microtubules at 21 °C on Tubulin Concentration. It has been observed above (Figures 3 and 5) that the measured rate of depolymerization of GDP–microtubules varied with tubulin concentration. Within the endwise disassembly model, the rate of dissociation, k_-^{21} ([M]), is proportional to the average concentration [M] of microtubule ends. It has been shown that the pseudo-first-order rate constant for the formation of microtubules, $k_+^{37}{}_{obsd} = k_+^{37}$ ([M]), varies cooperatively with tubulin concentration (Carlier & Pantaloni, 1978), and we demonstrated above that the average concentration of microtubule ends, [M], did not vary in the time course of our experiments. Consequently, the ratio of the observed rate of depolymerization at 21 °C to the apparent polymerization rate constant $k_+^{37}{}_{obsd}$ should be independent of [M], i.e., of the average length of microtubules. Evidence for this predicted behavior is shown in Table I, in which the polymerization rate at 37 °C and depolymerization rate at 21 °C were measured at different tubulin concentrations, in a range where a strong cooperativity was exhibited by both parameters. However, the ratio $k_-^{21}{}_{obsd}/k_+^{37}{}_{obsd}$ was kept constant and equal to 2.24 ± 0.08 at all tubulin concentrations.

Exchangeability of GTP in the Microtubule. An interpretation of the above results may be that the conformation of assembled tubulin is different before and after GTP hydrolysis. The possibility for the E site properties to be affected too was therefore investigated. Since it has previously been demonstrated that GTP could be chased off the E site before hydrolysis and replaced by another GTP molecule (Carlier & Pantaloni, 1981), it was interesting to examine whether GTP could be replaced by GDP as well and if this exchange would induce the conformational change usually triggered by GTP hydrolysis. In this hypothesis, the replacement of GTP by GDP would result in a stabilization of the microtubule, and a lower rate of depolymerization at 21 °C would be expected. In a typical experiment (Figure 6) tubulin at a concentration of 19 μM was polymerized in the presence of 240 μM [γ-^{32}P]GTP, and the time courses of both the extent of assembly and GTPase activity were followed. In parallel samples, 1, 2, or 3 mM unlabeled GDP–Mg^{2+} was added at different times after the beginning of assembly: (1) at a time of 3 min when 80% of microtubules were formed whereas only 50% GTP had been hydrolyzed on polymerized tubulin; (2) at a time of 20 min when the steady state of P_i liberation was attained. A slight decrease in turbidity to a lower plateau (12–20% according to GDP–Mg^{2+} molarity) followed the addition of GDP, due to the partial endwise depolymerization of microtubules.

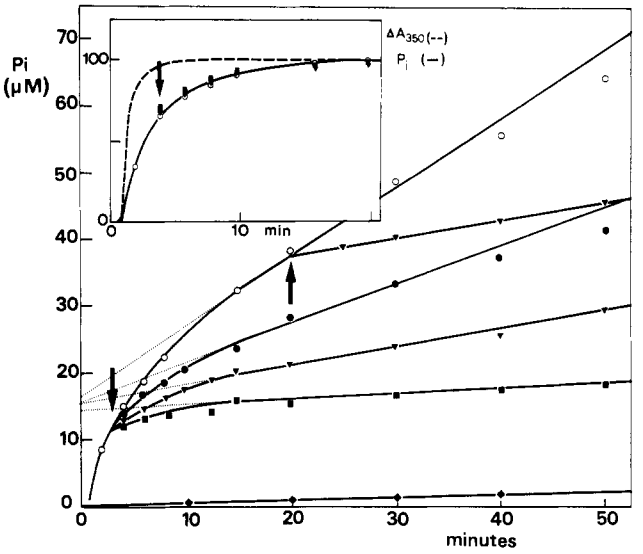

FIGURE 6: Effect of the addition of GDP–Mg^{2+} on GTPase activity at different times after assembly. The kinetics of P_i production during the assembly of 19 μM tubulin in the presence of 0.24 mM GTP and 6 mM Mg^{2+} was followed at 37 °C under different conditions: (O) no addition; (●) 1 mM GDP–Mg^{2+} was added at 3 min; (▼) 2 mM GDP–Mg^{2+} was added at 3 or 20 min; (■) 3 mM GDP–Mg^{2+} was added at 3 min; (♦) vinblastine (30 μM) was added at zero time. (Inset) Burst of GTP hydrolysis during tubulin assembly in the presence of added GDP–Mg^{2+} (same symbols). Data were obtained from total P_i production minus steady-state contributions for each concentration of added GDP–Mg^{2+}. Turbidimetric recording of microtubule formation is represented in the dashed line (normalized data).

When GDP–Mg^{2+} (1, 2, or 3 mM) was added in the early times of assembly, the rate of GTP hydrolysis slightly slowed down until a new inhibited steady state was attained. The same steady state was attained when GDP–Mg^{2+} was added at longer times (20 min).

All steady-state slopes (with or without added GDP–Mg^{2+}) were extrapolated to zero time. The extrapolated values, after taking into account the slight decrease of incorporated tubulin due to the 10–20% depolymerization following GDP–Mg^{2+} addition, yielded the same burst of one P_i hydrolyzed per molecule of incorporated tubulin. The time courses of GTP hydrolysis in the burst phases were obtained by subtracting the steady-state contributions from total hydrolysis (Figure

6, inset). Obviously, the kinetics of GTP hydrolysis in the microtubule appeared independent of GDP–Mg^{2+} added to the medium. These results indicate the GTP bound to the E site of polymerized tubulin could not be replaced by GDP before hydrolysis.

These results agree with the fact that addition of GDP–Mg^{2+} (1, 2, or 3 mM) to 3-min-old microtubules did not modify the time course of depolymerization at 21 °C induced 1 min later.

When added at steady state, GDP–Mg^{2+} (1–3 mM) inhibited GTP hydrolysis, which means that GTP bound to the E site on free tubulin and/or at the microtubule ends can be replaced by GDP. The observed 50% inhibition in 2 mM GDP and 0.2 mM GTP was in agreement with the relative affinities of the two nucleotides for pure dimeric tubulin in these medium conditions. These findings underline the difference in the conformation of the E site on assembled tubulin, depending upon tubulin location either in the body or at the end of the microtubule.

Discussion

Newly assembled microtubules depolymerize more rapidly than older microtubules. Three major explanations could account for this experimental result: (1) the appearance of a reaction product, due to GTP hydrolysis, for example, which would modify the medium in the time course of the assembly process; (2) a postassembly modification of the length distribution of microtubules involving a decrease in their number; (3) modification after assembly of the microtubule itself.

The first two hypotheses were discarded for the following reasons: first, the same phenomenon can be repeated after disassembly and reassembly in the same medium, and second, the microtubule length distribution was not modified during the time course of our experiments. The third hypothesis was considered, and a correlation between the decrease in the disassembly rate and other known modifications of microtubule properties was examined.

Previous results demonstrated the existence, during a certain time after assembly, of a transient gradient of the ratio [GTP]/[GDP] along the microtubules. Indeed, the newly polymerized tubulin, present at the end, carries essentially GTP while the previously assembled body of the microtubule is richer in GDP (Carlier & Pantaloni, 1981). We show that a tight correlation can be established between the GTP content of microtubules and their ability to depolymerize at 21 °C. Actually, we measured that when the burst phase of GTP hydrolysis accompanying assembly has been completed, the disassembly rate of tubulin is 2 times lower than the disassembly rate of GTP–tubulin from the microtubule. Therefore, GTP hydrolysis seems to increase the stability of microtubules.

It is interesting to compare our results with previous studies on microtubules polymerized in the presence of nonhydrolyzable GTP analogues (Arai & Kaziro, 1976; Weisenberg & Deery, 1976; Penningroth & Kirshner, 1977). Strong similarities were reported between microtubules assembled in the presence of GTP, GMP-PNP, and GMP-PCP. Comparison of thermal or Ca^{2+} stability of microtubules assembled in GTP or nonhydrolyzable GTP analogues showed that microtubules containing GDP, i.e., in which GTP had been hydrolyzed, depolymerized more rapidly than microtubules carrying GMP-PNP or GMP-PCP. We provide here evidence that microtubules carrying GTP depolymerize still more rapidly than microtubules–GDP. Fluorescence studies of Karr & Purich (1978) showed that under polymerizing medium conditions (37 °C) GTP and GDP stabilize different conformations of tubulin and that GMP-PNP or GMP-PCP mimics the binding of GTP. However, these experiments were performed with a tubulin concentration far below the critical concentration and do not provide information about the formation of tubulin inside the microtubule. Our results together with Weisenberg & Deery's (1976) and Karr et al.'s (1979) indicate that the conformation of the E site in the microtubule could be different according to whether GTP, GMP-PNP, or GDP is bound. We propose that hydrolysis of the γ-phosphate of GTP on the microtubule allows tubulin to dissociate from microtubule more slowly and not more rapidly as suggested before. The energy of GTP hydrolysis could contribute significantly to the dynamic stabilization of microtubule at steady state.

It should be pointed out that in the present experiments the two ends of the microtubules are undistinguishable. Actually, microtubules have a polar structure due to the polarity of the $\alpha\beta$ hetero dimer structure of tubulin. Therefore, different definitions can be proposed to differentiate the two ends, either from a structural point of view since there is a β end and an α end, from a cellular point of view, in which the distal end is distinguished from the basal end (near the centriole or the basal body or the microtubule organizing center), or from a kinetic point of view. In the latter case, the fast elongating end under pre-steady-state conditions may or may not be the same as the net assembly end at the steady state, as pointed out by Zeeberg et al. (1980). Thermodynamic considerations prescribe that the critical concentration of GTP–tubulin (and of GDP–tubulin) should be the same at the two ends, and so at the end which elongates faster with GTP–tubulin, GTP–tubulin will also dissociate faster. So the rate of dissociation of GTP–tubulin measured in our experiments is essentially the dissociation rate of the fastest elongating end. As for the rate of dissociation of GDP–tubulin, we cannot assess which one of the two ends is involved since it is not known whether the fastest elongating end is the same for GTP–tubulin and GDP–tubulin.

The presented results also bring some new information about the specificity of the E site for the different nucleotides in the microtubule. On soluble tubulin, it has been established that GTP is exchangeable and that the equilibrium dissociation constant for GDP is 3–6-fold larger than for GTP (Zeeberg & Caplow, 1979; Carlier & Pantaloni, 1978; Saltarelli & Pantaloni, 1982). During the delay between the incorporation of tubulin in the microtubule and the subsequent hydrolysis of GTP, it has been shown that GTP bound at the E site is freely exchangeable, and it was expected that unlabeled GDP, like unlabeled GTP, could displace [γ-^{32}P]GTP from the E site. However, we failed to exchange GTP for GDP on incorporated tubulin: this shows that, as soon as tubulin is assembled in the microtubule, the affinity of the E site for GDP decreases. This implies that the conformation of tubulin is already somehow modified as soon as it is incorporated in the microtubule and before GTP has been hydrolyzed. In addition, the configuration of the E site is again modified as GDP becomes blocked after hydrolysis. Schematically these results suggest that the E site can exist in at least three conformational states: (1) on soluble tubulin, the E site binds either GTP or GDP with comparable affinities; (2) on polymerized tubulin before hydrolysis, it binds GTP only; (3) on polymerized tubulin, after hydrolysis, the E site carries GDP only.

Our results indicate that microtubules are more stable in the third state, GDP–tubulin dissociating faster from the microtubule than GTP–tubulin. Another consequence of this work is that GTP–tubulin dissociation cannot be neglected in models proposed for microtubule assembly, as it has previously

been (Wegner, 1976; Weisenberg, 1980; Cote & Borisy, 1981).

Added in Proof

We are indebted to Drs. Engelborghs and Van Houtte, who communicated to us their manuscript (*Biophys. Chem.*, in press) while this paper was being processed for publication. These authors investigated the relaxation process of microtubules at steady state after a temperature jump from 35 to 25 °C and demonstrated that the relaxation time was proportional to the reciprocal of the elongation rate constant k_+ at 25 °C. In their experiments the perturbation of the equilibrium was small, as evidenced by the absence of change in the microtubule number during the relaxation process. Our experimental conditions differ from Engelborghs and Van Houtte's, since a substantial depolymerization of microtubules is observed upon the temperature jump from 37 to 21 °C consistently with a large change in microtubule number. Therefore, the kinetic process here observed was analyzed in terms of a true depolymerization of microtubules, differing from a relaxation, and the derived rate constant is essentially the dissociation rate constant k_- at 21 °C. This is corroborated by the fact that very similar data were obtained when the initial dissociation rate was analyzed instead of the first-order rate constant.

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