Assembly of Microtubule Protein: Role of Guanosine Di- and Triphosphate Nucleotides†

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ABSTRACT: A quantitative analysis of the interplay between guanosine 5′-triphosphate (GTP) and guanosine 5′-diphosphate (GDP) in microtubule assembly and accompanying GTP hydrolysis has been performed when tubulin was polymerized in the presence of microtubule-associated proteins (MAPs) which display an interfering GTPase activity. The use of adenylyl β-imidodiphosphate, which specifically inhibits the MAPs GTPase activity, and of vinblastine (or podophyllotoxin), which specifically inhibits GTP hydrolysis due to tubulin, made possible a study of the exclusive GTP hydrolysis associated to microtubule assembly. The results indicate that GDP binds to microtubule ends with an affinity comparable to GTP, thus strongly inhibiting both the elongation process and the steady-state GTP hydrolysis at microtubule ends. GDP shifts the equilibrium between tubulin and microtubules toward disassembly. The MAPs which are released from the microtubules during the GDP-driven depolymerization cluster on the remaining microtubules. The resulting increased stability of microtubules is quantitatively consistent with the decrease in the critical concentration of the polymerizing species GTP–tubulin.

The purpose of this work is to investigate the role of guanosine 5′-triphosphate (GTP)[1] and GDP in the regulation of microtubule assembly and steady state, under the most physiological conditions, i.e., using tubulin isolated through cycles of assembly–disassembly which contains specific microtubule-associated proteins (MAPs) known to stimulate assembly in vitro (Sloboda et al., 1976; Murphy et al., 1977). Early reports pointed to the inhibiting effect of GDP in microtubule assembly (Gaskin et al., 1974; Weisenberg et al., 1976). In a previous work (Carlier & Pantaloni, 1978), we explored the role of GTP and GDP in the assembly of pure tubulin dimers in the presence of 3.4 M glycerol and millimolar concentrations of magnesium ions, according to Lee & Timasheff (1975). Under these conditions, we demonstrated, and this was confirmed by other workers (Karr et al., 1979; Zackroff et al., 1980), that GDP promoted elongation of microtubules if not nucleation, and microtubules were stable in the presence of GDP, the equilibrium dissociation constant being twice larger than that in the presence of GTP. In contrast to these results indicating that the GDP–tubulin complex was able to participate in microtubule assembly, puzzling findings were obtained when tubulin was polymerized at low magnesium ion concentrations in the presence of MAPs and in the absence of glycerol (Weisenberg et al., 1976; Zackroff et al., 1980). An irreversible behavior of microtubules in GDP solutions then was observed, GDP blocking elongation and stabilizing microtubules in a metastable state. These features were inconsistent with the existing nucleation–condensation model (Oosawa & Kasai, 1962) currently admitted for microtubule assembly and prompted the authors to propose a model involving the cooperative addition of MAPs–tubulin oligomers in microtubule assembly (Weisenberg, 1980), which could account for the data obtained in the presence of GDP and colchicine (Deery & Weisenberg, 1981).

The interest in the role of GTP and GDP in microtubule regulation was further raised by the observation of subunit flow through microtubules (Margolis & Wilson, 1978), a phenomenon which is made thermodynamically possible by the energy supply (Wegner, 1976) which here can be brought on by the GTP hydrolysis accompanying the addition of GTP–tubulin to microtubules. The treadmilling process was arrested by GDP (Deery & Weisenberg, 1981; Margolis, 1981), which confirmed the role of GTP and GDP in the regulation of the steady state in microtubules. An analysis of GTP hydrolysis accompanying pure tubulin dimer assembly has been performed (Carlier & Pantaloni, 1981); however, the same study was more difficult when tubulin was polymerized from a whole microtubule protein solution which is known to contain an ATPase activity able to hydrolyze GTP in a concomitant way with polymerizing tubulin (Kirkpatrick et al., 1970; Ihara et al., 1979; Hiebsch et al., 1979; Maury, 1982). A comprehensive view of the relationships between GTP hydrolysis at microtubule ends and maintenance of the steady-state subunit flow through the microtubules requires the quantitative knowledge of the interplay between GTP and GDP in the different reactions involved in the mechanism of assembly. A preliminary approach to this problem is presented in this work. The nucleotide dependence of the elongation process and of steady-state GTP hydrolysis, the incorporation of [^{3}H]GTP in the microtubule in the presence of GDP, and the depolymerization of microtubules by GDP have been studied.

Materials and Methods

Reagents. 2-(*N*-Morpholino)ethanesulfonic acid (Mes) was purchased from Calbiochem; GDP, GTP, and adenylyl β-imidodiphosphate (AMPPNP) were from Boehringer; ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2-mercaptoethanol, vinblastine, and podophyllotoxin were from Sigma. [8-^{3}H]- and [γ-^{32}P]guanosine 5′-triphosphate were from Amersham.

Tubulin Purification. Microtubule protein was purified from fresh pig brain essentially by the method of Shelanski et al. (1973) with a slight modification (Fellous et al., 1978) of the polymerization buffer which consisted of 0.1 M Mes,

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[1] Abbreviations: MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)ethanesulfonic acid; AMPPNP, adenylyl β-imidodiphosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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pH 6.4, 1 mM EGTA, 0.5 mM GTP, 1 mM 2-mercaptoethanol, and 4 M glycerol. The protein was stored in 8 M glycerol at −25 °C after two cycles of assembly performed in this buffer and routinely used within 2 weeks following the preparation. A third cycle was performed the day the protein was used, in the same buffer. The microtubules were collected by centrifugation at 100000*g* for 30 min at 25 °C. The pellets were resuspended at 0 °C in standard buffer consisting of 0.1 M Mes, pH 6.6, and 0.25 mM $Mg(CH_3CO)_2$, and the solution was clarified by centrifugation at 20000*g* for 30 min at 0 °C. The microtubule protein was further deprived of glycerol and free nucleotides by gel filtration through Sephadex G25 equilibrated in standard buffer. The concentration of protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard, and also spectroscopically by using an extinction coefficient of 1.2 mg^{-1} mL cm^{-1} for the microtubule protein in the absence of nucleotides. After gel filtration, nucleotides were immediately added to tubulin at the desired concentrations, and the solutions were kept on ice for 30–60 min before the beginning of the experiment.

Polymerization Measurements. Microtubule assembly in standard buffer containing nucleotides at the indicated concentrations was monitored turbidimetrically at 350 nm by using a Beckman Acta V recording spectrophotometer equipped with a thermostated, jacketed 100-μL cell of 0.5-cm light path connected with a slow T-jump apparatus (temperature half raising time of 10 s).

The amount of microtubules formed was determined by both turbidimetric and direct measurements of the weight concentration of polymer sedimented at 37 °C. Polymerization of microtubules was recorded for a given time followed by depolymerization at 4 °C. The extent of reversible polymerization corresponding to microtubule formation was noted as the difference in the absorbances read at 37 and 4 °C after depolymerization. At the same time, microtubules were sedimented in the airfuge at 160000*g* for 3 min. Parallel samples were cooled and then centrifuged at 0 °C. The amount of microtubules formed at the time considered was calculated from the difference between the concentrations of the supernatants of the cold and warm centrifugations. Both the turbidimetric and sedimentation determinations of microtubules took into account the formation of nonmicrotubular aggregates which do not disassemble at low temperature. With this correction, a linear correlation was established between the amount of sedimented microtubules and the change in absorbance from 37 to 4 °C in a protein concentration range of 0–3 mg/mL. An extinction coefficient of 0.030 ± 0.005 μM^{-1} cm^{-1} at 350 nm was determined, a value in good agreement with that of Zackroff et al. (1980).

GTP Hydrolysis Measurements. GTP hydrolysis accompanying microtubule polymerization and the steady state was monitored by extraction of $^{32}P_i$ liberated from [γ-^{32}P]GTP, according to Avron (1960) and with the details previously described (Carlier & Pantaloni, 1981).

Incorporation of Labeled Nucleotides in Microtubules. Incorporation of [^{3}H]GTP in microtubules was measured by counting the radioactivity present in the material sedimented in the airfuge, as previously described (Carlier & Pantaloni, 1981).

Separation of Tubulin and MAPs. MAPs were separated from tubulin by phosphocellulose chromatography according to Weingarten et al. (1975). No more than 2 mg of microtubule protein was deposited on a 20-mL phosphocellulose column. Elution of tubulin was performed at 4 °C with 25 mM Mes, pH 6.6, containing 0.065 mM $Mg(CH_3CO_2)_2$ (a 4-fold dilution of standard buffer). The MAPs were then eluted with 0.8 M NaCl in the same buffer. The elution pattern was recorded at 280 nm with a ISCO UV-2 monitor.

Polyacrylamide Gel Electrophoresis in the Presence of $NaDodSO_4$. Discontinuous $NaDodSO_4$–polyacrylamide gel electrophoresis of microtubule protein was performed according to Laemmli (1970) except the running gel was a 4–11% acrylamide gradient. The slab gels, 0.7 mm thick, were electrophoresed for 18 h at 60 V. The slab was stained with Coomassie blue in methanol–water–acetic acid (454:454:92) and destained in the same solvent. A quantitative analysis was performed by scanning the gels with a Joyce microdensitometer.

Results

Inhibition of GTP Hydrolysis due to MAPs in Microtubule Protein. When microtubule protein was assembled at 37 °C in standard buffer containing 0.25 or 0.45 mM GTP, an important and fast consumption of GTP was measured. Figure 1A shows that 95% of the added GTP was hydrolyzed during the first 10 min of the assembly, at these two GTP concentrations. Furthermore, almost the same time course of GTP hydrolysis was observed in the presence of podophyllotoxin or vinblastine at concentrations which totally inhibited the assembly process and the subsequent GTP hydrolysis on tubulin. It was concluded that most of the GTP was hydrolyzed independently of tubulin assembly by a nucleotidase present in the preparation, thus presumably masking GTP hydrolysis due to tubulin assembly. The presence of an ATPase in cycled microtubule preparations from brain has already been reported (Kirkpatrick et al., 1970; Ihara et al., 1979), and this activity has putatively been attributed to the high molecular weight (M_r 350000) MAP_1 (Hiebsch et al., 1979; Maury, 1982).

The addition to the reaction medium of 1 mM unlabeled ATP–Mg as a competitor notably inhibited the hydrolysis of [γ-^{32}P]GTP, thus confirming that the high level of GTP hydrolysis observed was not due to tubulin which does not bind ATP. An even more pronounced inhibition was obtained with 1 mM AMPPNP–Mg present in the reaction mixture. It was verified that AMPPNP did not change the rate or the extent of polymerization or the critical concentration as compared to those obtained in the presence of 1 mM GTP, which indicated that tubulin assembly occurred independently of the GTP hydrolysis due to the MAPs. Figure 1B shows that the presence of 1 mM AMPPNP allows the measurement of exclusive GTP hydrolysis accompanying tubulin polymerization. The residual hydrolysis of GTP due to the MAPs in the presence of AMPPNP was determined by adding 100 μM podophyllotoxin or 25 μM vinblastine to the reaction mixture in order to prevent any tubulin assembly and subsequent hydrolysis of GTP. A low rate of P_i production by the MAP ATPase was then measured. This background could not be neglected but could be easily subtracted from the signal obtained in the absence of the drug. Since AMPPNP and GTP bind to this ATPase in a competitive manner, the residual GTP hydrolysis due to the MAPs represented a fraction of the overall GTP hydrolysis which varied with the concentration of GTP used in the experiments. In the range of 0.1–0.3 mM GTP routinely used, this fraction was 10–30% of the total initial rate of GTP hydrolysis and was measured for each experiment in the presence of vinblastine.

GTP Hydrolysis Accompanying Microtubule Formation in the Presence of MAPs. The correlation between microtubule assembly and associated GTP hydrolysis was studied in the presence of AMPPNP. Treatment of the data, once corrected for the residual GTP hydrolysis due to the MAPs, yielded

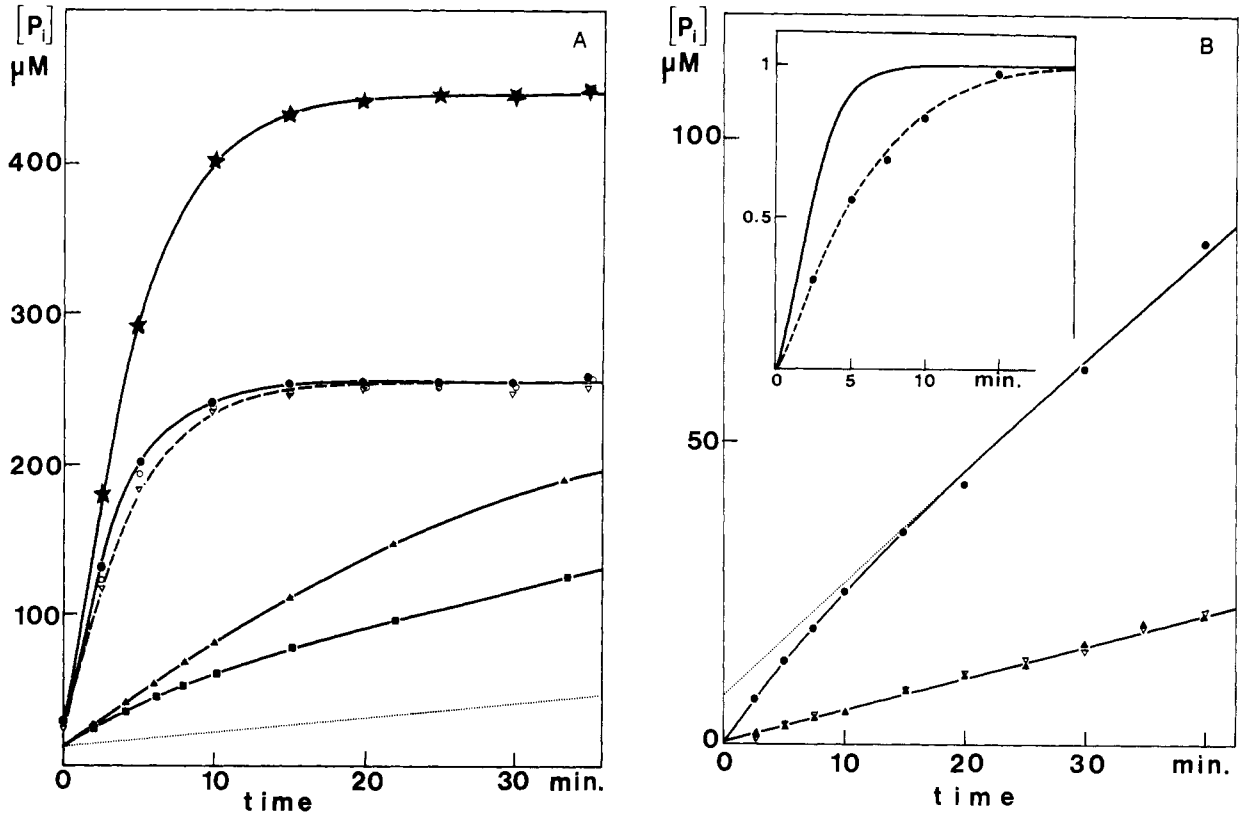

FIGURE 1: (A) GTP hydrolysis accompanying microtubule assembly in standard buffer. Tubulin at a concentration of 25 μM was polymerized in standard buffer containing 0.45 mM [γ-^{32}P]GTP (★) or 0.25 mM [γ-^{32}P]GTP and the following additions: (●) none; (○) 50 μM podophyllotoxin; (▽) 35 μM vinblastine (dashed line); (▲) 1 mM ATP–Mg; (■) 1 mM AMPPNP–Mg (i.e., buffer A). The dotted line at the bottom represents the virtual residual GTP hydrolysis in the presence of 1 mM AMPPNP–Mg plus podophyllotoxin or vinblastine. (B) GTP hydrolysis accompanying tubulin assembly in buffer A. Tubulin at a concentration of 15.5 μM was polymerized in buffer A containing 0.25 mM [γ-^{32}P]GTP (●). In parallel samples, 15 μM vinblastine (▲) or 100 μM podophyllotoxin (▽) was added to inhibit microtubule assembly and the accompanying GTP hydrolysis. Inset: The solid line represents the evolution of turbidity and the dashed line the liberation of P_i during the burst phase derived from the curve (●). Data are normalized to 1 mol of tubulin polymerized according to Carlier & Pantaloni (1981).

results qualitatively similar to those previously obtained in the case of pure tubulin assembly in the presence of Mg^{2+} ions and glycerol (Carlier & Pantaloni, 1981). In this medium too, a burst of roughly one GTP molecule hydrolyzed per molecule of tubulin dimer incorporated in the microtubule was determined. GTP was hydrolyzed in the body of microtubules in a kinetic process independent of tubulin polymerization and following microtubule assembly monitored by turbidimetry (Figure 1B, inset). The burst of GTP hydrolyzed during assembly was followed by a linear steady-state P_i production as already observed in the case of pure tubulin assembly, and which has been attributed to GTP hydrolyzed at microtubule ends (David-Pfeuty et al., 1978).

A thorough investigation of the involvement of GTP and GDP in microtubule steady-state and associated GTP hydrolyses requires the knowledge at all times of the amount of both nucleotides and of the rate of GTP hydrolysis at microtubule ends. This condition can be experimentally fulfilled provided that AMPPNP is present in the assembly buffer. The next experiments related in this work will therefore be performed in buffer A which is standard buffer in which 1 mM AMPPNP–Mg is included.

Inhibition of the Steady-State Rate of GTP Hydrolysis at Microtubule Ends by GDP. Microtubule protein, at a concentration of 2–2.5 mg/mL, was polymerized at 37 °C in buffer A containing 250 μM [γ-^{32}P]GTP. At time 15 min, when the steady-state rate of GTP hydrolysis was reached, GDP at a series of concentrations was added to the samples. In control samples run in parallel, both vinblastine (25 μM) and GDP were added simultaneously at time 15 min to measure the residual GTP hydrolysis due to MAP_i under the same conditions. The time course of [γ-^{32}P]GTP hydrolysis was measured in all samples, and the rate of GTP hydrolysis in each sample as well as the real concentrations of remaining GTP at time 15 min and total GDP (i.e., the sum of GDP produced at time 15 min and of added GDP) was determined. The rate of P_i production following the addition of GDP was measured. The corresponding control rate measured in the presence of vinblastine was subtracted in order to obtain the intrinsic rate of GTP hydrolysis at the ends of microtubules at each GDP concentration. A linear Dixon plot of the reciprocal of the rate of GTP hydrolysis vs. GDP concentration was derived from the data. The same experiment was performed at three concentrations of GTP. Apparent inhibition dissociation constants (K_I^{app}) for GDP were derived from each Dixon plot (Figure 2). The intrinsic inhibition constant, as derived from the plot of K_I^{app} vs. [GTP], was very low, and the slope of this plot indicated that the affinity of GTP for microtubule ends was 1.6-fold higher than that of GDP. These results show that the rate of GTP hydrolysis at microtubule ends is dependent on the [GTP]/[GDP] ratio only, at all concentrations of GTP and GDP.

Another way to observe the inhibition of steady-state GTP hydrolysis at microtubule ends is to follow the decrease in the rate of GTP hydrolysis as GDP accumulates with time. This experiment was done at different initial GTP concentrations. The lower the GTP concentration, the sooner the slopes of P_i liberation decayed. The rates of P_i production were measured at different times as the tangents to these curves and corrected for the interfering GTPase activity of MAPs measured in

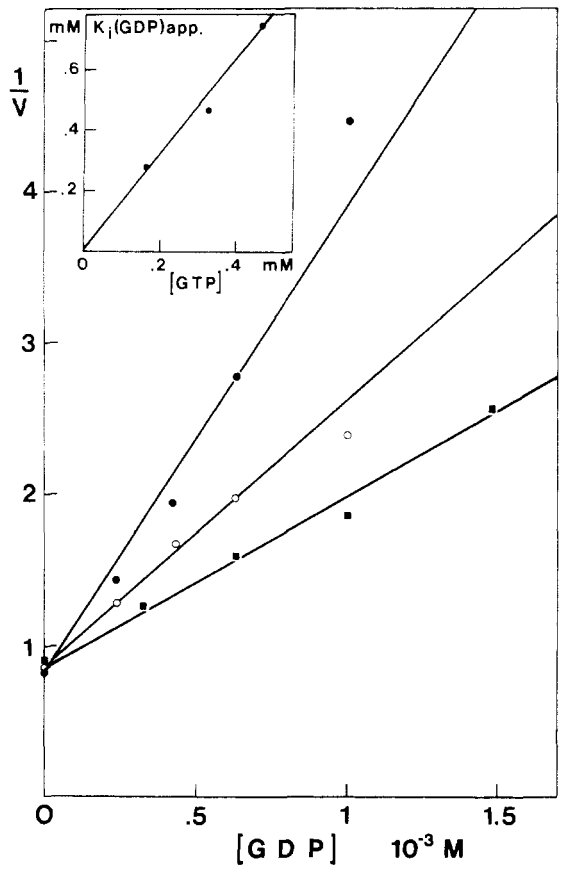

FIGURE 2: Inhibition of steady-state GTP hydrolysis by GDP. Several samples of tubulin (22 μM) were polymerized in buffer A containing 0.20 (■), 0.35 (○), and 0.50 mM (●) [γ-^{32}P]GTP, and the time course of GTP hydrolysis was followed. At time 15 min (when 0.165, 0.315, and 0.465 mM GTP, respectively, remained in solution), GDP was added at different concentrations to each sample, and GTP hydrolysis was followed after GDP addition. In parallel samples, 25 μM vinblastine and the same respective amounts of GDP were added at time 15 min, in order to determine the contribution of the MAPs to the total GTP hydrolysis. The reciprocal value of the corrected steady-state rate of GTP hydrolysis at microtubule ends was plotted vs. the total GDP concentration present at time 15 min. Inset: Plot of the apparent inhibition constant for GDP vs. GTP concentration.

parallel samples containing vinblastine. The amounts of GTP and GDP present in the medium at the same time were determined. It was found that the rates of GTP hydrolysis were the same at different absolute concentrations of GTP and GDP, provided that the [GTP]/[GDP] ratio was the same. Again these data (not shown) indicated that the rate of GTP hydrolysis was dependent on the [GTP]/[GDP] ratio only.

Inhibition of Microtubule Elongation by GDP. Previous experiments with pure tubulin demonstrated that GDP was unable to promote nucleation of microtubules, but elongation of preformed seeds was possible in the presence of 1–2 mM GDP and the total absence of GTP (Carlier & Pantaloni, 1978). The same experiment was performed by Zackroff et al. (1980) with a tubulin preparation containing MAPs, and the same result was obtained, the rate constant for the addition of GDP–tubulin to microtubules being 60-fold lower than that for GTP–tubulin. On the other hand, when GDP was added during GTP-supported assembly, conflicting results and interpretations were reported: Karr et al. (1979) determined a slow incorporation of GDP in the microtubule while Zackroff et al. (1980) observed the net arrest of growth following the addition of GDP, the level of polymerization attained depending on the time at which GDP was added. With the purpose of understanding the reason for these discrepancies, the GTP-supported elongation of microtubules was studied in buffer A in the presence of different concentrations of GDP.

Microtubules were formed at 37 °C from a microtubule protein solution in buffer A containing 0.100 mM GTP. The growth step which was followed turbidimetrically is a first-order process, as already analyzed by Johnson & Borisy (1977) on a quite similar microtubule preparation, thus indicating that the microtubule number concentration (M) was kept constant during the whole elongation reaction. GDP was added at time 40 s (a time at which the average length of the microtubules was less than 0.5 μm), and the evolution of turbidity was recorded until a stable plateau was reached. The first-order rate constant measured for the exponential process following GDP addition decreased with increasing concentrations of added GDP, as well as the amount of polymer formed at equilibrium. A preliminary control was performed in order to check that the inhibition of elongation by GDP added at the early times of assembly was not due to a fast depolymerization by GDP of a number of nuclei, which would decrease the number of microtubules M. If we assume that each microtubule carries n elongating sites, a proportional decrease in the value $k_+m = k_+nM$ of the elongation first-order rate constant would therefore be expected. That this was not the case was assessed by two measurements: (1) the rate of endwise depolymerization of microtubules at 4 °C was the same whether microtubules were elongated in the absence (sample 1) or in the presence (sample 2) of 1 mM GDP, which shows that the number concentration of microtubules was the same; (2) In the two samples, the observed elongation first-order rate constants (k_1 and k_2), the weight amounts of microtubules at equilibrium (C_{w1} and C_{w2}), and their average lengths (l_1 and l_2) were measured. Values of 1.6 and 1.9 were found respectively for the ratios l_1/l_2 and C_{w1}/C_{w2} and 3.5 for k_1/k_2. In the hypothesis of a disappearance of nuclei due to GDP addition, the ratio of the average lengths would be expected to be $l_1/l_2 = (C_{w1}/C_{w2})(k_2/k_1) = 0.54$. There is a large discrepancy between this number and the measured ratio, which better agrees with the value of 1.9 expected if the number of microtubules does not change upon addition of GDP. In a parallel experiment, GTP hydrolysis accompanying assembly was measured following the addition of GDP at time 40 s and was inhibited in a parallel fashion to elongation. Figure 3A shows that roughly the same amount of microtubules was obtained at equilibrium whether GDP was added to microtubules totally formed in GTP exclusively or to microtubules initiated in GTP, provided that this amount was measured when the same concentrations of both GTP and GDP were present in the two samples. The exact concentrations of GTP and GDP were derived from the records of GTP hydrolysis performed on parallel samples. This observation indicated that the final extent of polymerization reached reflected an equilibrium state of the microtubules which was characterized by the same critical concentration in both samples.

Within the simple model currently admitted, microtubule elongation is described by

$$-\frac{d(C - C_c)}{dt} = k_+m(C - C_c) \qquad (1)$$

where C is the tubulin dimer concentration, m is the elongating sites concentration, C_c is the tubulin critical concentration, and k_+ and k_- are the association and dissociation rate constants.

The measured pseudo-first-order elongation rate constant is $k_{obsd} = k_+m$. The evolution of k_{obsd} with GDP was studied at different concentrations of GTP. Double-reciprocal plots of $k_{obsd}(0) - k_{obsd}([GDP])$ vs. [GDP] were linear and extrapolated, at infinite concentrations of GDP, to a value

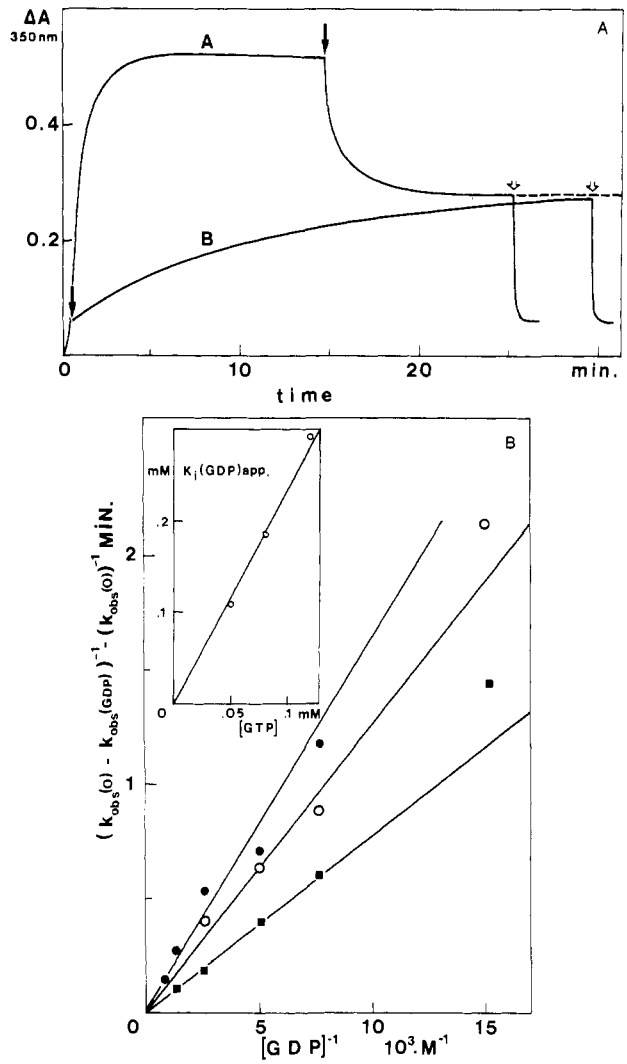

FIGURE 3: Depolymerization and inhibition of the elongation of microtubules by GDP. (A) Time course of tubulin assembly followed turbidimetrically. Tubulin (30 μM) was polymerized in buffer A containing 0.100 mM GTP. In sample A, 1.6 mM GDP was added at time 15 min (solid arrow), and the depolymerization of microtubules was followed. In sample B, the same amount of GDP was added at 40 s (solid arrow). In parallel samples, the disappearance of [γ-^{32}P]GTP was measured. When the samples were cooled (open arrows), the [GDP]/[GTP] ratios were 1.64/0.06 = 27 for sample A and 1.62/0.075 = 22 for sample B. (B) Double-reciprocal plot of the change in the observed first-order elongation rate constant of microtubules with GDP added at the early times of the assembly process. The experiment was conducted as described in Figure 3A, at three different initial concentrations of GTP: 50 (■), 80 (○), and 120 μM (●). Inset: Plot of the apparent inhibition constant for GDP vs. GTP concentration.

$k_{obsd}(0) - k_{obsd}(\infty)$ which was, within 5%, equal to the value $k_{obsd}(0)$ measured in the absence of GDP (Figure 3B). This result indicated that no notable elongation of microtubules occurred at saturating concentrations of GDP. This conclusion agrees with the data of Zackroff et al. (1980), who found, in independent experiments, almost 2 orders of magnitude difference between the elongation rate constants of GTP–tubulin and GDP–tubulin. From the double-reciprocal plots shown in Figure 3B, apparent equilibrium inhibition constants for GDP were derived at different concentrations of GTP (Figure 3B, inset). The data indicate that the intrinsic inhibition dissociation constant is very low as compared to the concentrations used and that GTP bound to microtubule ends with an affinity about 2-fold higher than GDP, a value which is in reasonable agreement with the value found for the inhibition of GTP hydrolysis at microtubule ends.

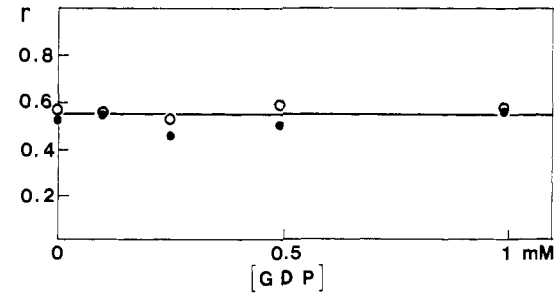

FIGURE 4: Incorporation of [^{3}H]GTP in microtubules elongated in the presence of GDP. The experimental procedure is described in Figure 3A, except GTP was labeled. The samples A_i (○), to which GDP was added after assembly at time 15 min, and B_i (●), in which microtubules were elongated in the presence of GDP, were centrifuged together at time 30 min and processed for the determination of the [^{3}H]GTP content of microtubules. The number of molecules of [^{3}H]GTP incorporated per molecule of tubulin polymerized (r) is plotted vs. [GDP] added to the samples.

In order to determine whether GDP was incorporated in the microtubules elongated in the presence of GDP, the experiment shown in Figure 3B was reproduced at different concentrations of GDP, and by using [^{3}H]GTP. The microtubules were sedimented at time 30 min when the equilibrium was reached, and the radioactivity content of the pellets was examined as described under Materials and Methods. At all GDP concentrations assayed, no difference was found in the ^{3}H-labeled nucleotide content between samples completely polymerized in [^{3}H]GTP and to which GDP was added at equilibrium and corresponding samples in which microtubules were elongated in the presence of the same concentration of GDP. Figure 4 shows that in all cases the same amount (0.55) of [^{3}H]GDP bound per molecule of tubulin incorporated in the microtubule was found. This result confirms that the observed inhibition of elongation due to GDP was not caused by a slower incorporation of GDP–tubulin but rather was the result of a classical competitive inhibition, in which GDP binding to microtubule ends prevented further addition of GTP–tubulin to microtubules.

Depolymerization by GDP of Microtubules Assembled in the Presence of GTP. A limited depolymerization of microtubules upon the addition of GDP has been reported (Weisenberg et al., 1976; Zackroff et al., 1980). In GDP solutions, the change in absorbance vs. tubulin concentration yielded a straight line of a shallower slope than that of the GTP control (Zackroff et al., 1980). These results were interpreted within a decrease in the fraction of polymerizable tubulin in the presence of GDP. We repeated this experiment and confirmed this observation. The depolymerization of microtubules by GDP at different concentrations and at a given tubulin concentration (10–15 μM) was then studied under conditions where the concentrations of GTP and GDP were monitored. The change in turbidity upon addition of GDP was recorded, and the increase in the free tubulin concentration was determined by protein measurement of the supernatant of sedimented microtubules. The extent of depolymerization increased with increasing GDP concentration. ADP did not replace GDP, which eliminated the possibility of a trivial metal-chelating effect of GDP causing the observed depolymerization. GDP was added in all samples at the same time after the beginning of assembly, routinely after 15 or 20 min. The samples were centrifuged 15 min later and processed for the determination of microtubules and soluble tubulin. The determination of GTP hydrolysis was done in parallel samples in which the same amounts of GDP were added at the same times. The concentrations of free nucleotides were derived

Table I: Equilibrium Parameters of Microtubules upon Depolymerization by GDP[a]

expt	added [GDP] (μM) at t = 15 min	[GDP] (μM) at t = 30 min	[GTP] (μM) at t = 30 min	[GDP]/[GTP] (μM)	C_s (μM)	[T-GTP] (μM)	[T-GDP] (μM)
I	0	60	47.2	1.27	8.7	6.0	2.7
	83	136	53.8	2.52	8.7	4.6	4.1
	166	219	54.4	4.02	9.2	3.8	5.4
	332	381	58.3	6.5	10.3	3.1	7.2
	662	705	63.6	11	10.6	2.15	8.45
	990	1028	68.8	14.9	11.1	1.76	9.34
II	0	98	193	0.51	8.2	6.9	1.3
	166	257	200	1.3	8.8	6.0	2.8
	332	415	207	2.0	9.2	5.4	3.8
	662	739	214	3.45	9.7	4.3	5.4
	990	1066	215	4.96	10.0	3.6	6.4
	1320	1393	218	6.39	10.4	3.2	7.2

[a] Tubulin at a concentration of 12.6 μM was polymerized at 37 °C in buffer A containing 117 μM GTP (experiment I) or 301 μM GTP (experiment II). GDP was added at time 15 min at the indicated concentrations. Microtubules were sedimented in the airfuge at time 30 min, and the concentration of tubulin in the supernatant (C_s) was determined. The determination of the amount of GTP hydrolyzed at time 30 min in each sample was made in parallel. The concentrations of GTP and GDP present at the time of centrifugation are derived. The concentrations of free T-GTP and T-GDP are calculated as indicated in the text.

from the hydrolysis plots. A slight correction was made for GDP bound to microtubules but could be neglected since the concentrations of nucleotides were in a range of at least 1 order of magnitude higher than the tubulin concentration.

From the measurement of the total free tubulin concentration in the supernatant(C_s), and by using the value of 1/2.8 previously determined for the ratio α of the dissociation constants K_T and K_D of GTP–tubulin and GDP–tubulin complexes, respectively (Zeeberg & Caplow, 1979), the concentrations of free GTP–tubulin and GDP–tubulin at a given [GDP]/[GTP] ratio could be calculated as follows:

$$[\text{T–GTP}] + [\text{T–GDP}] = C_s \tag{2}$$

$$\frac{[\text{T–GDP}]}{[\text{T–GTP}]} = \alpha\frac{[\text{GDP}]}{[\text{GTP}]} \tag{3}$$

$$[\text{T–GTP}] = \frac{C_s}{1 + \alpha([\text{GDP}]/[\text{GTP}])}$$

$$[\text{T–GDP}] = \frac{C_s\alpha([\text{GDP}]/[\text{GTP}])}{1 + \alpha([\text{GDP}]/[\text{GTP}])} \tag{4}$$

where $\alpha = K_T/K_D$ and $K_T \ll [\text{GTP}]$ and $K_D \ll [\text{GDP}]$.

These equations indicate that the concentrations of T–GTP and T–GDP only depend on the ratio of the concentrations of free GTP and GDP. Two series of experiments were performed at two different initial GTP concentrations. Table I summarizes the data. A plot of $[\text{T–GDP}]^{-1}$ vs. $([\text{GDP}]/[\text{GTP}])^{-1}$ yielded a single straight line for the two experiments (Figure 5). This plot extrapolated at high [GDP]/[GTP] ratios to a value of [T–GDP] equal to the total concentration of tubulin (as determined from the protein concentration in the supernatant of the cold centrifugation). This result indicated that at infinite concentrations of GDP, the microtubules were totally depolymerized by GDP. The same result was repeatedly obtained at different tubulin concentrations.

Calculation of the concentrations of unpolymerized T–GTP and T–GDP (Table I) demonstrated that concomitantly with microtubule depolymerization upon addition of GDP the concentration of T–GTP decreased. Since the experiments related above were understood within a nonparticipation of the GDP–tubulin in microtubule assembly, the partial concentration of T–GTP in the supernatant of sedimented microtubules is an actual measure of the critical concentration of the microtubules remaining in solution after partial depolymerization by GDP. That this T–GTP concentration decreases with increasing depolymerization is indicative of a higher stability of the remaining microtubules. This higher stability is suggestive of a clustering of the MAPs along the remaining microtubules. This explanation supports the observation that the depolymerization process following GDP addition was qualitatively initially as fast as the depolymerization induced by dilution (a process in which MAPs are diluted to the same extent as microtubules) but grew slower and slower and needed 10–15 min to reach completion. Such an observation corroborates the findings that the MAPs decreased the rate of disassembly of microtubules (Murphy et al., 1977). The hypothesis of an increased binding of MAPs to microtubules remaining after partial depolymerization by GDP was then tested in three different experiments in which

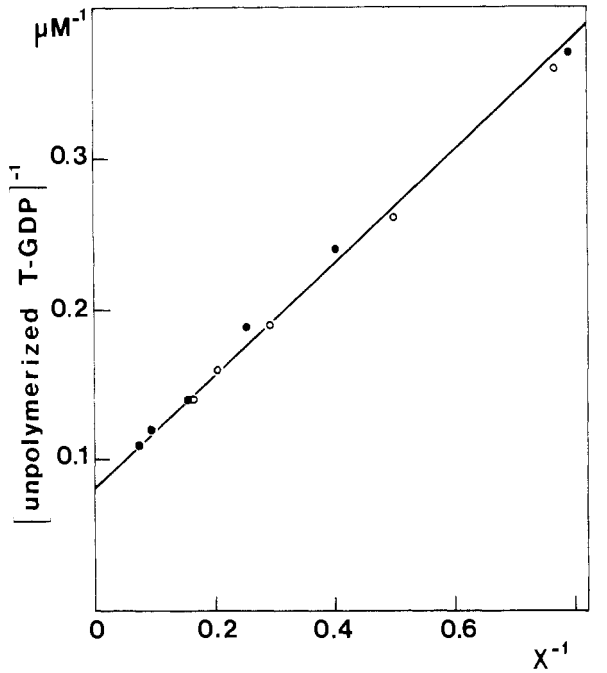

FIGURE 5: Depolymerization of microtubules by GDP. Several samples of tubulin (12.6 μM) were polymerized in the presence of 117 (●) and 301 μM (○) GTP, and GDP at different concentrations was added at time 15 min. The samples were centrifuged at time 30 min, and the concentration of tubulin in the supernatant was determined. The data processing is shown in Table I. The evolution of unpolymerized T–GDP concentration with increasing [GDP]/[GTP] ratios (X) is plotted in a double-reciprocal plot which extrapolates at a concentration of 12.2 μM at high concentrations of GDP, indicating a total depolymerization of microtubules at saturating GDP concentration.

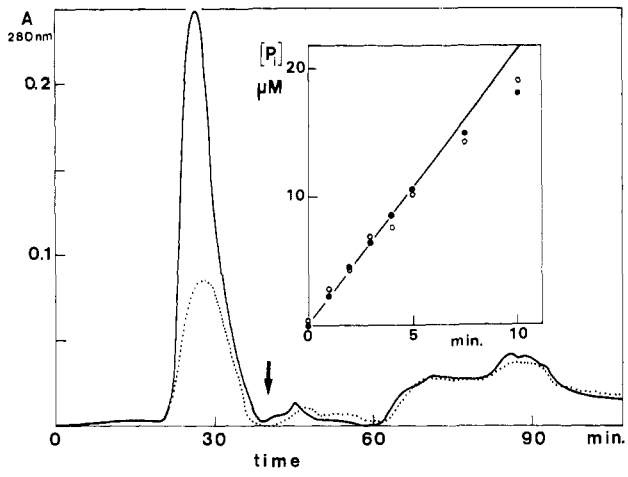

FIGURE 6: Elution pattern of the phosphocellulose chromatography of microtubules in equilibrium with GTP and partially depolymerized by GDP. The pellets of microtubules polymerized with 1 mM GTP (solid line) and partially depolymerized by 1 mM GDP (dashed line) were resuspended and chromatographed on two identical phosphocellulose columns eluted at the same flow rate of 0.35 mL/min. The first peak represents tubulin. At the time indicated by the arrow, 0.8 M NaCl was added to the column buffer in order to elute the MAPs in the second peak. Inset: Time course of GTP hydrolysis due to the MAPs cosedimenting with microtubules polymerized with 1 mM GTP (●) and partially depolymerized by 1 mM GDP (○). The GTP hydrolysis was assayed on the pellets resuspended in standard buffer containing 0.3 mM [γ-^{32}P]GTP and 30 μM vinblastine.

the microtubule content in MAPs before and after addition of GDP was directly determined.

(1) Microtubule protein (6.4 mg/mL) was polymerized in buffer A containing 0.25 mM GTP for 15 min. The solution was then divided in two samples of 1.2 mL. GTP (1 mM) was added to the first sample (A) and GDP (1 mM) to the second one (B). The extent of depolymerization (43%) was checked turbidimetrically. At 30 min, the two samples were layered on a 50% sucrose cushion in buffer A containing no guanosine nucleotides and centrifuged at 100000g, 30 °C, for 1 h. The pellets were resuspended in standard buffer diluted 4-fold and deposited on the top of two identical 2 cm × 6 cm phosphocellulose columns which were run at identical flow rates by using a LKB multichannel peristaltic pump. The elution of tubulin and MAPs was performed as described under Materials and Methods. The elution patterns obtained for the A and B samples are shown in Figure 6. The areas under the tubulin peaks were 32 and 15 arbitrary units, respectively, which agrees with the previously estimated depolymerization. In contrast, mostly identical profiles were obtained for the elution of the MAPs, indicating, as expected, that the [MAPs]/[tubulin] ratio on the microtubules increases after their partial depolymerization by GDP.

(2) The nucleotide triphosphatase activity indicative of MAP_1 concentration was measured on the A and B microtubule pellets centrifuged as above on a sucrose cushion and resuspended in the same final volume of standard buffer containing 0.3 mM [γ-^{32}P]GTP and 25 μM vinblastine. The inset of Figure 6 shows that exactly the same GTPase activity was measured in the two samples, showing that despite the fact that half the amount of tubulin was present in the pellet of microtubules partially depolymerized by GDP, the same amount of MAP_1 was present.

(3) $NaDodSO_4$–polyacrylamide gel electrophoresis was performed on the two pellets of A and B samples as described under Materials and Methods. The same amounts of τ factor and of MAP_1 and MAP_2 were found in A and B samples, while the tubulin band was twice as intense in A as in B. This last result corroborates the two others and emphasizes the reliability of the proposed model according to which the amount of MAPs bound to microtubules increases as they depolymerize upon GDP addition.

If we assume, in a first approximation supported by the experiments related above, that the MAPs bind to microtubules with a high affinity, then the concentration of free MAPs is buffered by microtubules, and the relative increase in the microtubule content in MAPs upon depolymerization by GDP is directly proportional to the ratio of the weight concentrations of microtubules in the absence and in the presence of GDP, [MT(0)]/[MT(GDP)]. It is well-known that the usual distribution of MAPs on the microtubule wall is one MAP molecule every 12 tubulin dimers (Amos, 1977), when microtubules are formed from a solution of microtubule protein prepared by a cycling procedure. The binding of MAPs confers a supplementary stabilization to microtubules, so that the equilibrium association constant K_c of these microtubules is dependent on the number (j) of molecules of MAPs bound per i molecules of tubulin incorporated in the microtubule according to (Pantaloni et al., 1981)

$$K_c = K_c^* \gamma^{j/i} \tag{5}$$

where γ is the stabilization constant of one molecule of tubulin in the microtubule by one molecule of MAP and K_c^* is the equilibrium association constant of microtubules in the absence of MAPs. In previous experiments, the value of γ was determined from the change in K_c upon the addition of pure tubulin dimers to microtubule protein, which resulted in a decrease in the j/i ratio (Pantaloni et al., 1981). In the present experiments, the j/i ratio is increased by depolymerization of microtubules at a constant concentration of MAPs. With the assumption of a value of 1/12 for j/i in the absence of GDP, the value of j/i in the presence of GDP could be calculated as $j/i = (1/12)([MT(0)]/[MT(GDP)])$. The corresponding equilibrium association constant was the reciprocal of the equilibrium concentration of GTP–tubulin calculated above (Table I).

The semilogarithmic plot of the change in $[T\text{–}GTP]^{-1} = K_c$ vs. j/i was a straight line of slope $\log \gamma = 5.88$ (Figure 7A). This value of $10^{5.88}$ found for γ is in good agreement with the previous determination of 10^6 obtained from a study of the destabilization of microtubules due to MAP depletion (Pantaloni et al., 1981).

Another way of describing these results is to express the extent of microtubule depolymerization with increasing [GDP]/[GTP] ratio. In this view, a combination of eq 4 and 5 gives

$$K_c = \frac{1 + \alpha([GDP]/[GTP])}{C_s} = K_c^* \gamma^{j/i} \tag{6}$$

with

$$\frac{j}{i} = \left(\frac{j}{i}\right)_0 \frac{C_0 - C_{c_0}}{C_0 - C_s}$$

in which C_0 is the total concentration of tubulin, C_s is the concentration of unpolymerized tubulin measured at a given [GDP]/[GTP] ratio, $(j/i)_0 = 1/12$ is the number of MAPs per molecule of polymerized tubulin in the absence of free GDP, and C_{c_0} is the critical concentration measured in the absence of GDP. By definition $1/C_{c_0} = K_c^* \gamma^{(j/i)_0}$ and eq 6 can be written

$$\frac{1 + \alpha([GDP]/[GTP])}{C_s} = \frac{1}{C_{c_0}} \gamma^{(j/i)_0 (C_s - C_{c_0})/(C_0 - C_s)}$$

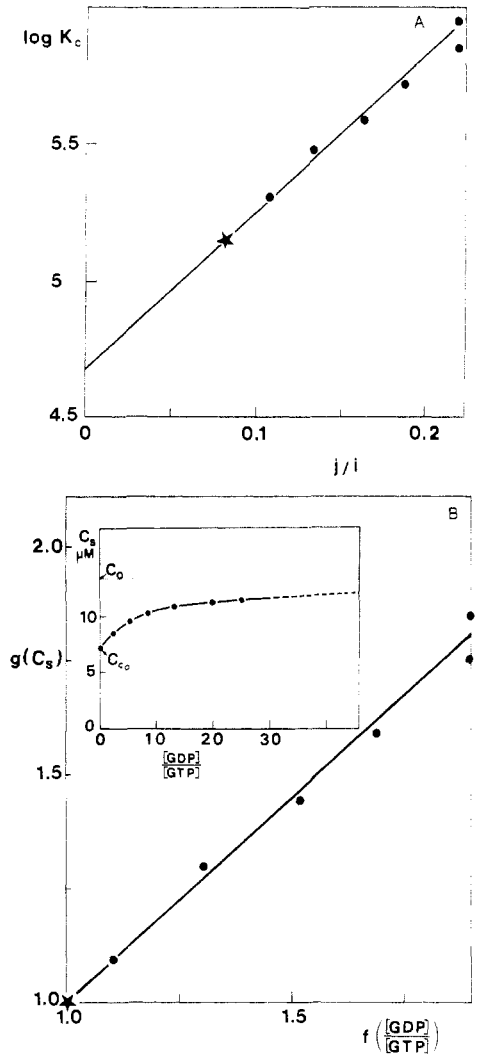

FIGURE 7: Stabilization of the microtubules by a higher density of MAPs bound following the partial depolymerization by GDP. (A) Logarithmic plot of the equilibrium association constant of microtubules, K_c, vs. the number j of MAP molecules bound per i molecules of polymerized tubulin. K_c is calculated as the reciprocal of the unpolymerized T–GTP concentration at equilibrium. A reference value of 1/12 was assumed for j/i in the absence of GDP (★). From this reference value, the increase in j/i upon addition of GDP was calculated as described in the text. (B) Plot of the linear correlation between $\log \{C_{c_0}[1 + \alpha([GDP]/[GTP])]\} = f([GDP]/[GTP])$ and $\log C_s + (j/i)_0(C_s - C_{c_0})/(C_0 - C_s) \log \gamma = g(C_s)$ which relates the extent of depolymerization of microtubules, measured by the concentration of total unpolymerized tubulin C_s, to the [GDP]/[GTP] ratio. The data are those of Figure 7A. A value of 1/12 was taken for $(j/i)_0$, 5.88 for $\log \gamma$, and 1/2.8 for α. Inset: A direct plot of C_s vs. [GDP]/[GTP].

The following relation between a function g (C_s) of the concentration of unpolymerized tubulin C_s and a function f ([GDP]/[GTP]) can be derived:

$$\log \left\{ C_{c_0} \left(1 + \alpha \frac{[GDP]}{[GTP]} \right) \right\} = \log C_s + \left(\frac{j}{i} \right)_0 \frac{C_s - C_{c_0}}{C_0 - C_s} \log \gamma \tag{7}$$

In this equation, all the parameters can be experimentally determined. The linear correlation of g vs. f obtained in a plot of the data according to eq 7 exhibited a slope of 0.9, which is very close to the expected value of 1.0 (Figure 7B).

Discussion

The study of GTP hydrolysis associated with tubulin assembly in the presence of microtubule-associated proteins has been made possible by inhibiting the interfering GTPase activity due to the MAPs, which is about 10-fold higher than the tubulin assembly dependent GTP hydrolysis in fresh microtubule protein preparations, and subsequently introduces a leak of GTP in the tubulin–microtubule system. Advantage was taken of the exclusive specificity of tubulin for GTP and of the wide base specificity of the MAPs ATPase for triphosphate nucleotides. The ATP analogue AMPPNP does not bind to tubulin or modify the parameters of the equilibrium between tubulin and microtubules but strongly inhibits the MAP GTPase activity. This inhibition is of a competitive type and thus depends on the respective concentrations of GTP and AMPPNP. When the GTPase activity of MAPs was inhibited by 95%, GTP hydrolysis due to tubulin assembly represented the major part of the overall liberation of P_i, and the contribution of the MAPs could easily be subtracted. AMPPNP was preferred to ATP, with which side reactions of phosphate transfer can occur: actually, cycled microtubule preparations have been shown to display a nucleoside diphosphokinase activity (Jacobs & Huitorel, 1979) which can catalyze the following reactions in the presence of unlabeled ATP and γ-^{32}P-labeled GTP:

$$ATP + GDP \rightarrow ADP + GTP$$
$$ADP + GTP^* \rightarrow ATP^* + GDP$$

The net result is to make γ-^{32}P-labeled ATP and to decrease the specific radioactivity of GTP. Consequently, the observed liberation of $^{32}P_i$ can be due to a complex sum of the reactions of hydrolysis of ATP and GTP having time-dependent specific radioactivities. This can explain that the apparent inhibition observed in Figure 1 with ATP was lower than that with AMPPNP: this latter compound cannot be hydrolyzed or used in a phosphate-transfer reaction.

The results obtained concerning the competitive interplay between GTP and GDP in steady-state GTP hydrolysis at microtubule ends and in microtubule elongation can both be best explained within a model in which the nucleotides freely exchange at microtubule ends. The obtainment of a linear plot of $1/v$ vs. [GDP] indicates that the ends can be considered as catalytic centers which hydrolyze GTP. Microtubule ends bind GTP and GDP in a competitive fashion with almost identical affinities. The rate of GTP hydrolysis only depends on the [GTP]/[GDP] ratio, which indicates that the ends carry GTP or GDP but are never empty in the range of nucleotide concentrations used. GDP binding to microtubule ends results in the inhibition of elongation, tubulin being unable to bind to a microtubule elongating site carrying GDP. It is noteworthy that the inhibiting effect of GDP in microtubule assembly is not only a passive role which consists of decreasing the concentration of the polymerizable species GTP–tubulin: in such a simple model, no inhibition of the apparent elongation rate constant k_+m would be observed. Although it is not notably incorporated in the microtubules, GDP does play an active part by binding strongly to microtubule ends. In this respect, GDP behaves like colchicine, as first proposed by Deery et al. (1978), GDP blocking the ends for further elongation but dissociating freely from the ends to be replaced by GTP. The following equations describe the model, which is very similar to the model developed by Lambeir & Engelborghs (1980), for the binding of the tubulin–colchicine complex to microtubule ends:

$$\begin{aligned} m\text{–}GTP + T\text{–}GTP &\underset{k_-}{\overset{k_+}{\rightleftharpoons}} m\text{–}GTP \\ m\text{–}GTP + T\text{–}GDP &\rightleftharpoons m'\text{–}GDP \\ m\text{–}GTP + GDP &\rightleftharpoons m'\text{–}GDP + GTP \end{aligned} \tag{8}$$

m–GTP represents an elongating site carrying GTP at microtubule ends and m′–GDP a nonelongating site blocked by GDP. Within this model, exogenous GDP cannot be incorporated in the microtubules. The elongation reaction is

$$-\frac{d(C - C_c)}{dt} = k_+ m(C - C_c)$$

where C is the concentration of unpolymerized T–GTP and m the concentration of m–GTP. The observed first-order elongation rate constant is $k_{obsd} = k_+ m$. The total concentration of end sites is $m_0 = m + m'$. Consequently

$$m = \frac{m_0}{1 + \beta([GDP]/[GTP])} \quad (9)$$

where β is the ratio of the equilibrium dissociation constants of GTP and GDP, respectively, from microtubule ends.

In the presence of GDP, the apparent first-order elongation rate constant is

$$k_{obsd} = k_+ m_0[1 + \beta([GDP]/[GTP])]^{-1} \quad (10)$$

This equation accounts for the data shown in Figure 3B. It should be pointed out that this model does not discriminate whether GDP or T–GDP binds to microtubule ends to inhibit the elongation process. These two cases are actually formerly the same since they both satisfy eq 9 and 10. The fact that the GTP–tubulin complex cannot bind to an elongating site unless it has GTP bound exclusively compares favorably with the previous finding of a gradient of GTP on the elongating microtubules (Carlier & Pantaloni, 1981) and supports the hypothesis of a steady-state cap of GTP at the ends of the microtubules (M. F. Carlier and D. Pantaloni, unpublished experiments).

The studies of microtubule depolymerization upon addition of GDP corroborate this model in which GDP–tubulin does not participate in the GTP-promoted assembly. Microtubules depolymerized more and more upon addition of increasing amounts of GDP, and extrapolation of the data to high concentrations of GDP indicated that they can be virtually totally disassembled. The fact that a limited depolymerization was observed at [GDP]/[GTP] ratios of about 10–20 is explained by the stabilization brought about by the clustering of MAPs on microtubules partially depolymerized by GDP. Consequently, the T–GTP critical concentration can decrease to less than micromolar amounts and nevertheless maintain the microtubule equilibrium. The number j of MAPs molecules bound per i polymerized tubulin dimers increases with the dissociation of microtubules upon addition of GDP. A value as high as 1 MAP per 4.5 tubulins was obtained experimentally, which corresponds to a 6-fold decrease in the critical concentration.

This model accounts for the qualitative observations of Zackroff et al. (1980), who found that microtubules depleted in MAPs are more sensitive to GDP. Furthermore, the derived numerical value of the parameter γ accounting for the stabilizing effect of MAPs compares favorably with the number found in previous experiments.

As pointed out by one of the referees of this paper, the clustering of MAPs on the microtubules might also explain the recently observed stabilization of cellular microtubules by azide in vivo (Bershadsky & Gelfand, 1981).

We did not observe the irreversible behavior of microtubules in GDP reported by Weisenberg et al. (1976) and Zackroff et al. (1980). However, it is possible that the observations made by these authors are explained within the model proposed here. Indeed, although no data are available about the rate at which GTP was consumed in their experiments, the high microtubule protein and low GTP (0.1 mM) concentrations used anticipate a very strong GTP hydrolysis due to MAPs, and the resulting fast time-dependent change in the [GDP]/[GTP] ratio may be responsible for the apparent nonequilibrium state observed. The addition of a high amount of GDP in the time course of assembly may result in an apparent arrest of elongation due to the binding of GDP to microtubule ends.

The last point to be discussed is the difference of behavior in the presence of GDP whether microtubules are assembled from pure tubulin in the presence of glycerol and high magnesium ion concentrations or from a cycled whole microtubule protein preparation in the absence of glycerol. In the first case, a true thermodynamic equilibrium between microtubules and GDP–tubulin was possible, and a critical concentration of GDP–tubulin was established in solution; in contrast, in the latter case studied here, no stabilization of microtubules was possible in GDP solutions, indicating that the critical concentration of GDP–tubulin was out of the range of the investigated tubulin concentrations. Although we do not have any definite explanation for these different behaviors, the strong stabilizing effect of glycerol and magnesium ions may allow the participation of GDP–tubulin in microtubule assembly. In addition, the microtubular lattice may not be exactly the same for these two types of microtubules, which may affect their relative stabilities.

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Reactions of β-(2-Furyl)propionyl Coenzyme A with "General" Fatty Acyl-CoA Dehydrogenase†

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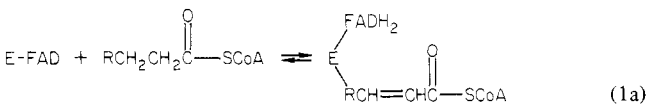
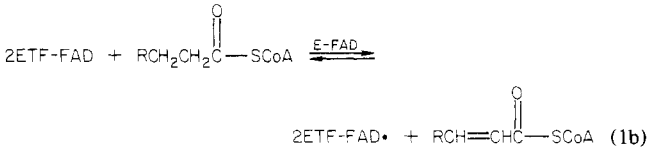
ABSTRACT: We have prepared a new pseudosubstrate for the "general" acyl-CoA dehydrogenase, β-(2-furyl)propionyl-CoA (FPCoA). This substrate reacts with enzyme to yield *trans*-β-(2-furyl)acryloyl-CoA (FACoA) which absorbs maximally at 340 nm, the isosbestic point for oxidized and fully reduced flavin. FPCoA is a better substrate (lower K_m, higher V_{max}) than butyryl-CoA but not as good a substrate as octanoyl-CoA. By observing the rate of formation of FACoA and comparing it with the rate of formation of the semiquinone of electron-transfer flavoprotein (ETF), we have established a 2:1 stoichiometry for this reaction (see eq 1b). The reaction of FPCoA with acyl-CoA dehydrogenase is a biphasic first-order reaction when either flavin reduction or FACoA formation is observed. However, observation of FACoA production reveals a new oxygen-dependent production of enoyl-CoA product which is not reflected in the reaction profile of the FAD of fatty acyl-CoA dehydrogenase. This reaction requires oxygen, produces H_2O_2, and can therefore be characterized an "oxidase" reaction. The reaction is zero order and is linearly dependent upon enzyme concentration. The charge transfer product complex of FACoA and acyl-CoA dehydrogenase is not stable and completely dissociates, as evidenced by the complete disappearance of the charge transfer electronic band with a first-order rate constant of 2×10^{-2} s^{-1}. The zero-order rate constant characterizing the production of FACoA is also 2×10^{-2} s^{-1}. Therefore, oxygen reacts with reduced acyl-CoA dehydrogenase at the rate of dissociation of the charge transfer product complex. Another electron acceptor, crotonyl-CoA, reacts in a transhydrogenation reaction at this same rate of charge transfer product complex dissociation. It is significant that the normal electron acceptor, ETF, reacts with a much larger rate constant of $\simeq 30$ s^{-1}. This establishes the fact that normal electron transfer to ETF occurs within the charge transfer product complex. These data would seem to establish that the charge transfer product complex formed upon reduction of acyl-CoA dehydrogenase with saturated fatty acyl-CoA substrate is essential in assuring the transfer of electrons to ETF in preference to electron transfer to oxygen or enoyl-CoA substrate.

Fatty acyl-CoA dehydrogenase (pig liver, general enzyme) is an FAD-containing enzyme responsible for the oxidation of saturated fatty acyl-CoA esters and the transfer of electrons to a protein substrate, electron transfer flavoprotein (ETF) (Crane & Beinert, 1956), as shown in eq 1.



(1a)



(1b)

The reaction shown in eq 1a shows biphasic kinetics and a very large primary isotope effect (Reinsch et al., 1980a). The reaction in Figure 1b also shows a primary isotope effect (J. Schmidt, J. Reinsch, and J. T. McFarland, unpublished results) and consists of the reaction of ETF to yield an anionic semiquinone at a rate which is sufficiently fast to be consistent with the overall process of β-oxidation (Reinsch et al., 1980b). In order to correlate the rate of C=C formation with flavin reduction, we have synthesized β-(2-furyl)propionyl-CoA (FPCoA), a pseudosubstrate, which produces a product, *trans*-β-(2-furyl)acryloyl-CoA (FACoA), absorbing at 340 nm. Since the flavin species involved in the enzyme reaction of Figure 1a have an isosbestic point at 340 nm, this substrate allows us to determine the absorbance change occurring during double-bond formation.

Experimental Procedures

Reagents and Enzyme. The enzyme and ETF purifications have been described previously (McKean et al., 1979).

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