Interrelationships of Tubulin–GDP and Tubulin–GTP in Microtubule Assembly

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ABSTRACT: We previously reported that direct incorporation of GDP (i.e., without an initial hydrolysis of GTP) into microtubules occurs throughout an assembly cycle in a constant proportion. The exact proportion varied with reaction conditions, becoming greater under all conditions in which tubulin–GDP increased relative to tubulin–GTP (low Mg^{2+} and GTP concentrations, high tubulin concentrations, and in the presence of exogenous GDP). These findings led us to explore further interrelationships of tubulin–GDP and tubulin–GTP in microtubule assembly. We have now determined the minimum amount of tubulin–GTP required for the initiation of microtubule assembly and the relative efficiency with which tubulin–GDP participates in microtubule elongation. When GTP, GDP, and tubulin concentrations were varied at a constant Mg^{2+} concentration (0.2 mM), initiation of assembly required that 35% of the nucleotide-bearing tubulin be in the form of tubulin–GTP, and incorporation of tubulin–GDP into microtubules during elongation was only 60% as efficient as would be predicted on the basis of its proportional concentration in the reaction mixtures. Very different results were obtained when the Mg^{2+} concentration was varied. Even though Mg^{2+} enhances the binding of GTP to tubulin (the equilibrium constant for the exchange of GTP for GDP was 0.2 in the absence of exogenous Mg^{2+}, 3 with 0.2 mM Mg^{2+}, 5 with 0.5 mM Mg^{2+}, and 11 with 2 and 4 mM Mg^{2+}), as Mg^{2+} was increased the proportion of tubulin–GTP required for the initiation of microtubule assembly rose greatly, and the direct incorporation of tubulin–GDP into microtubules during elongation became progressively more efficient. In the absence of exogenous Mg^{2+}, only 20% tubulin–GTP was required for initiation, and tubulin–GDP was directly incorporated into microtubules half as efficiently as would be predicted on the basis of its concentration in the reaction mixture. At the highest Mg^{2+} concentration examined (4 mM), 80% tubulin–GTP was required for initiation of assembly, and tubulin–GDP was incorporated into microtubules as efficiently as tubulin–GTP.

Tubulin, the major protein component of microtubules, has two binding sites for guanine nucleotides (Weisenberg et al., 1968; Bryan, 1972). One of them, termed the nonexchangeable site, contains nucleotide which cannot be removed from tubulin without denaturing the protein, while the other, termed the exchangeable site, accommodates nucleotide which can be displaced by exogenous GDP or GTP. Generally, GTP bound at the exchangeable site of the protein is required to initiate the assembly of microtubules, and in the reaction process, this GTP is hydrolyzed to GDP (Kobayashi, 1975; Weisenberg et al., 1976; Penningroth & Kirschner, 1977; David-Pfeuty et al., 1977; MacNeal & Purich, 1978).

GDP, on the other hand, is usually an inhibitor of tubulin polymerization (Arai & Kaziro, 1977; Carlier & Pantaloni, 1978; McNeal & Purich, 1978; Jameson & Caplow, 1980; Zackroff et al., 1980), but its potency varies greatly with reaction conditions (Huang et al., 1985b; Duanmu et al., 1986; Hamel et al., 1986a). Nevertheless, incorporation of tubulin–GDP onto microtubule seeds has been described (Carlier & Pantaloni, 1978; Karr et al., 1979; Zackroff et al., 1980), and a number of workers have reported at least partial stability of steady-state microtubules in GDP (Weisenberg et al., 1976; Margolis, 1981; Zeeberg & Caplow, 1981), which indicates that tubulin–GDP can add to microtubule ends. More recently, Manser and Bayley (1985) reported substantial direct incorporation of GDP into microtubules when assembly was induced with guanosine 5′-(β,γ-methylenetriphosphate) and when tubulin partially depleted of exchangeable nucleotide by alkaline phosphatase treatment was induced to polymerize with pyrophosphate (Bayley & Manser, 1985).

We had previously reported that significant amounts of tubulin–GDP can copolymerize with tubulin bearing GTP in the exchangeable site in reactions induced by glutamate (Hamel et al., 1984) or glycerol (Duanmu et al., 1986). Most recently, we extended this observation to microtubule-associated protein-dependent (MAP-dependent)[1] microtubule assembly (Hamel et al., 1986b). Using a heat-treated MAP preparation and electrophoretically homogeneous tubulin bearing [8-^{14}C]GDP in the exchangeable site (a system with negligible nucleoside diphosphate kinase and nonspecific phosphatase contamination), we observed direct incorporation of GDP (i.e., without an initial hydrolysis of GTP) into microtubules in a relatively constant proportion throughout an assembly cycle. The exact amount of GDP directly incorporated into microtubules varied with reaction conditions, but the proportion increased under all conditions in which tubulin–GDP would have increased relative to tubulin–GTP—at low Mg^{2+} concentrations (Huang et al., 1985b), at low GTP concentrations (with the tubulin concentration held constant), at high tubulin concentrations (with the GTP concentration held constant), and if exogenous GDP was added to the reaction mixture (Hamel et al., 1986b). Since GTP was an absolute requirement for assembly under all conditions examined, these findings were consistent with earlier reports that GTP is particularly required for the initiation of polymerization, while tubulin with GDP in the exchangeable site can incorporate into microtubules during the elongation phase of the assembly reaction (Carlier & Pantaloni, 1978; Karr et al., 1979; Zackroff et al., 1980).

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[1] Abbreviations: MAPs, microtubule-associated proteins; Mes, 4-morpholineethanesulfonate.

These observations on the direct incorporation of GDP into microtubules led us to explore further the interrelationship of tubulin–GDP and tubulin–GTP in microtubule assembly. Here we examine two questions. First, what is the minimum ratio of tubulin–GTP relative to tubulin–GDP required for the initiation of microtubule assembly, and is it constant or does it vary under different reaction conditions? Second, since tubulin–GDP can be incorporated directly into microtubules, how does the efficiency of this reaction compare with that of the incorporation of tubulin–GTP?

Materials and Methods

Materials. Electrophoretically homogeneous calf brain tubulin and heat-treated MAPs were prepared as described previously (Hamel & Lin, 1984a; Duanmu et al., 1986). The tubulin and MAP preparations used here had negligible amounts of nucleoside diphosphate kinase and nonspecific phosphatase activities (Hamel & Lin, 1984a). The two tubulin preparations (with nonradiolabeled GDP or [8-^{14}C]GDP in the exchangeable site) both had 1.7 mol of guanine nucleotide bound/mol of tubulin, which was half GDP and half GTP. All protein preparations were chromatographed on Sephadex G-50 (superfine) to remove unbound nucleotide. All nucleotides (both radiolabeled, obtained from Moravek Biochemicals, and nonradiolabeled, obtained from Sigma) were repurified by ion-exchange chromatography on DEAE-Sephadex A-25. The nucleotide preparations have been described in detail previously (Hamel et al., 1986ab). Mes (free acid) was obtained from Sigma, and 1.0 M stock solutions were adjusted to pH 7.0 with NaOH. An ample supply of maytansine was a generous gift of Dr. M. Suffness, Natural Products Branch, National Cancer Institute.

Methods. All reaction mixtures contained 0.1 M Mes (pH 7.0) and $MgCl_2$, tubulin, nucleotides, heat-treated MAPs, and other components as indicated in individual experiments. Microtubule assembly was followed turbidimetrically (Gaskin et al., 1974) in a Gilford Model 250 recording spectrophotometer equipped with an electronic temperature controller. After base lines were established at 0 °C, at zero time the controller was set at 37 °C. Cold reversibility was routinely evaluated by resetting the temperature controller at 0 °C (indicated in figures by an arrow on the abscissa). The assembly threshold for any reaction component was defined as the concentration required for a cold-reversible change in turbidity less than 0.010 A_{350} unit.

The binding of radiolabeled GDP and GTP to tubulin and displacement of radiolabeled GDP from tubulin by nonradiolabeled GDP and GTP were measured by centrifugal gel filtration chromatography (Penefsky, 1977), as described in detail previously (Hamel & Lin, 1984b). Because the antimitotic drug maytansine potently inhibits both the binding and dissociation of nucleotides at the exchangeable site of tubulin (Huang et al., 1985a), 20 μM drug was added to reaction mixtures at the end of the incubation period (15 min at 0 °C unless otherwise specified). The Sephadex G-50 (superfine) syringe columns were prepared in solutions containing 0.1 M Mes (pH 7.0), 20 μM maytansine, and the same $MgCl_2$ concentration used in the reaction mixtures of the specific experiments. Radioactivity and protein (Lowry et al., 1951) were quantitated in the filtrates obtained by centrifugation, and data are expressed as moles of [8-^{14}C]GDP or [8-^{14}C]GTP per mole of tubulin. In a specific experiment, quadruplicate 0.1-mL aliquots of a reaction mixture were filtered through four syringe columns. At least three independent experiments were performed under each reaction condition presented. In all cases, mean values are presented in the tables and figures, and 84% of the experimental data points were within 15% of the mean values. Because of this variability, sufficient replicate experiments were performed under each condition so that the standard error of the mean was within 5% of the mean.

Analysis of the radiolabeled nucleotide content of a microtubule pellet was performed by quantitating protein and bound radiolabeled nucleotide following ultracentrifugation. The 0.25-mL reaction mixtures were incubated for 15 min at 37 °C and diluted with 2.0 mL of an isothermic solution of 50% sucrose (Terry & Purich, 1980) containing 0.1 M Mes (pH 7.0) and 0.2 mM $MgCl_2$. The samples were centrifuged at 40000 rpm for 30 min in a Beckman Ti50 rotor prewarmed to 37 °C. The pellet was washed 3 times with 2 mL of the isothermic sucrose solution and dissolved in 0.5 mL of 8 M urea. The radioactivity and protein content (Lowry et al., 1951) of the resulting solution were determined. Data were expressed as moles of [8-^{14}C]GDP per mole of tubulin [see Hamel et al. (1986b)]. A minimum of three values were obtained for each experimental condition. Reproducibility was comparable to that in the centrifugal gel filtration experiments.

Results

Proportion of Tubulin–GTP Required for the Initiation of Microtubule Assembly. Our observation that some tubulin–GDP was directly incorporated into microtubules throughout polymerization without an initial exchange of GDP for GTP (Hamel et al., 1986b)—even though GTP was required for an assembly reaction—led us to explore further the interrelationships of tubulin–GDP and tubulin–GTP in polymerization. In particular, we wanted a more precise idea of the relative amounts of tubulin–GDP and tubulin–GTP in a reaction mixture at the onset of assembly, and we wanted to investigate the four reaction components (GTP, GDP, tubulin, and Mg^{2+}) which had the most important effects on the direct incorporation of GDP into microtubules.

Although the exchange of GDP and GTP has long been known to be fast (Weisenberg et al., 1968; Caplow & Zeeberg, 1980), perhaps as a consequence of the rapid release of nucleotide from the exchangeable site (Brylawski & Caplow, 1983), the measurement of tubulin–GDP and tubulin–GTP in a reaction mixture presents a number of difficulties. An extremely useful technique is the centrifugal gel filtration method (Penefsky, 1977), first used with tubulin by Caplow and Zeeberg (1980), but it has two major drawbacks. First, it does not measure a true equilibrium, for significant nucleotide losses from tubulin can occur during the filtration procedure [discussed in detail in Hamel et al. (1984, 1986a) and in Huang et al. (1985ab)]. Second, tubulin polymers are not readily separated from unpolymerized tubulin during filtration, making specific quantitation of tubulin–nucleotide complexes in a postpolymerization reaction mixture impossible by this technique.

While no solution is yet available for the second problem, two recent observations from our laboratory now make the centrifugal gel filtration method useful for equilibrium measurements under selected reaction conditions. First, tubulin binds exchangeable-site nucleotide much more tenaciously at higher than at lower reaction pH values (Hamel et al., 1986a). Second, the antimitotic drug maytansine almost completely inhibits both the binding and release of nucleotides at the exchangeable site of tubulin (Huang et al., 1985a). Therefore, in the studies to be presented here, a reaction pH of 7.0 was used, and 20 μM maytansine was present throughout centrifugal gel filtration.

To evaluate the total nucleotide content of tubulin, two preparations of the protein were used which were identical in

Table I: Binding of GDP and GTP to Tubulin at 0 °C without MAPs and at 22 °C with MAPs[a]

nucleotide	pmol of nucleotide/μg of protein (mol/mol of tubulin)[e]	
	0 °C – MAPs	22 °C + MAPs
GDP[b]	2.20 (0.22)	1.86 (0.25)
GTP[c]	5.15 (0.52)	4.20 (0.56)
total	7.35 (0.74)	6.06 (0.81)
% GTP[d]	70	69

[a] Each 0.43-mL reaction contained 0.1 M Mes (pH 7.0), 0.5 mM $MgCl_2$, 20 μM (2.0 mg/mL) tubulin, 15 μM GTP, and 0.67 mg/mL heat-treated MAPs, as indicated. After 15 min at either 0 or 22 °C, as indicated, the samples were processed by centrifugal gel filtration as described in the text at the appropriate temperature. [b] Obtained from reaction mixtures containing tubulin initially bearing [8-^{14}C]GDP in the exchangeable site and nonradiolabeled GTP. [c] Obtained from reaction mixtures containing tubulin initially bearing nonradiolabeled GDP in the exchangeable site and [8-^{14}C]GTP. [d] Tubulin not bearing exchangeable nucleotide was not included in these calculations. [e] The correction assumes that the concentration of MAPs in the filtrates following centrifugal gel filtration was identical with the concentration of MAPs in the original reaction mixtures.

their nucleotide content and in their polymerization properties. First, residual, "endogenous" GDP was quantitated in reaction mixtures containing tubulin bearing [8-^{14}C]GDP in the exchangeable site together with nonradiolabeled, exogenous GTP and, if appropriate, GDP. Second, the binding of "exogenous" nucleotides to tubulin was quantitated in reaction mixtures containing tubulin originally bearing nonradiolabeled GDP together with the appropriate exogenous nucleotides (either [8-^{14}C]GTP alone, [8-^{14}C]GTP + nonradiolabeled GDP, or nonradiolabeled GTP + [8-^{14}C]GDP). The values obtained with these two tubulin preparations in any specific reaction condition were summed to yield the total amount of guanine nucleotide bound at the exchangeable site. Data presented elsewhere (Hamel et al., 1984, 1986a,b; Huang et al., 1985a,b) have documented that the GDP bound in similar tubulin preparations is fully exchangeable.

One final problem remained. Since tubulin–nucleotide equilibria are significantly affected by reaction pH (Hamel et al., 1986a) and Mg^{2+} concentration (Huang et al., 1985b), it seemed quite possible that reaction temperature or addition of MAPs to the reaction mixture could have major effects on the relative amounts of GDP and GTP bound by tubulin. Rapid tubulin polymerization and GTP hydrolysis, however, make it impossible to examine nucleotide binding at 37 °C in the presence of MAPs. Nevertheless, we could detect no significant effect of higher reaction temperature alone or of MAPs at temperatures up to 22 °C (room temperature)[2] on nucleotide binding under any reaction condition we have examined. Table I presents a detailed study with 0.5 mM $MgCl_2$ quantitating the relative amounts of GDP and GTP bound by 20 μM tubulin in the presence of 15 μM GTP. Approximately 70% of the exchangeable nucleotide bound by tubulin under this condition was GTP and 30% GDP, both at 0 °C – MAPs and at 22 °C + MAPs.[3] Therefore, because maytansine's

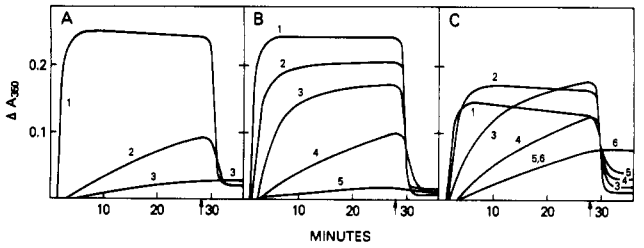

FIGURE 1: Microtubule assembly reactions as a function of concentrations of reaction components. Each 0.25-mL reaction mixture contained 0.1 M Mes (pH 7.0), 0.2 mM $MgCl_2$, heat-treated MAPs in a 1:3 weight ratio to the tubulin, and the indicated concentrations of tubulin, GTP, and GDP. At the times indicated by the arrows on the abscissa, the temperature control unit was set at 0 °C. (A) Assembly reactions with different concentrations of GTP. Each reaction mixture contained 20 μM (2.0 mg/mL) tubulin and GTP as follows: curve 1, 100 μM; curve 2, 8 μM; curve 3, 6 μM. (B) Assembly reactions with different concentrations of GDP. Each reaction mixture contained 100 μM GTP, 20 μM (2.0 mg/mL) tubulin, and GDP as follows: curve 1, none; curve 2, 100 μM; curve 3, 150 μM; curve 4, 300 μM; curve 5, 400 μM. (C) Assembly reactions with different concentrations of tubulin (and MAPs). Each reaction mixture contained 15 μM GTP and tubulin as follows: curve 1, 10 μM (1.0 mg/mL); curve 2, 20 μM (2.0 mg/mL); curve 3, 30 μM (3.0 mg/mL); curve 4, 40 μM (4.0 mg/mL); curve 5, 50 μM (5.0 mg/mL); curve 6, 60 μM (6.0 mg/mL).

inhibition of nucleotide exchange is more potent at 0 °C than at 22 °C (Huang et al., 1985a) and to conserve MAPs, throughout the studies presented here we have assumed that the equilibrium data[4] we have obtained at 0 °C without MAPs are equally valid just prior to the onset of microtubule assembly at 37 °C + MAPs.

We particularly wished to evaluate the GDP/GTP equilibrium under reaction conditions at which only minimal assembly occurred (polymerization "thresholds"). We found that the minimum concentration of GTP needed for polymerization varied with the Mg^{2+} concentration (see below), with the lowest GTP concentration associated with polymerization at 0.2 mM Mg^{2+} (exogenously added). Most of our studies were therefore performed at 0.2 mM Mg^{2+}, and Figure 1 presents assembly reactions (a complete cycle of polymerization at 37 °C and depolymerization at 0 °C) occurring at a variety of GTP, GDP, and tubulin concentrations.

Figure 1A contrasts assembly reactions with three concentrations of GTP at 20 μM tubulin (+0.67 mg/mL MAPs)—a brisk and extensive reaction at 100 μM GTP (curve 1); a delayed, slow, and depressed reaction at 8 μM GTP (curve 2); and essentially no polymerization at 6 μM GTP (curve 3), as indicated by the absence of a cold-reversible component to the small rise in turbidity observed at 37 °C and the similar sluggish turbidity change occurring in the absence of GTP (data not presented).

Figure 1B demonstrates the progressive inhibition of polymerization with 20 μM tubulin (+0.67 mg/mL MAPs) and 100 μM GTP as the GDP concentration was raised. With a GDP:GTP ratio of 3:1 (300 μM GDP, curve 4), partial assembly occurred, but with a GDP:GTP ratio of 4:1 (400 μM GDP, curve 5), polymerization was negligible.

Figure 1C presents experiments examining the effect of varying the tubulin (and MAPs) concentration with the GTP concentration held constant at 15 μM. The assembly pattern at this low GTP concentration changed substantially as the

[2] There is no significant assembly of microtubules with tubulin + heat-treated MAPs at temperatures below 25 °C (Hamel et al., 1984).

[3] Although Caplow and Zeeberg (1980) reported that nucleotide exchangeability was sharply reduced in ring oligomers isolated from microtubule protein, we have not observed any inhibition of nucleotide exchange in purified tubulin following the addition of MAPs. We have nonetheless observed abundant ring oligomers formed in our tubulin preparations in the presence of MAPs. The reason for this difference is not known but may indicate loss of a factor inhibiting the exchange reaction during our preparative procedure (Hamel & Lin, 1984a).

[4] We, as well as others (Caplow & Zeeberg, 1980), have found incubation time to have no effect on the values for tubulin-bound nucleotide obtained by the centrifugal gel filtration method. Thus, in the presence of maytansine, values obtained by this technique almost certainly represent quantities present at equilibrium.

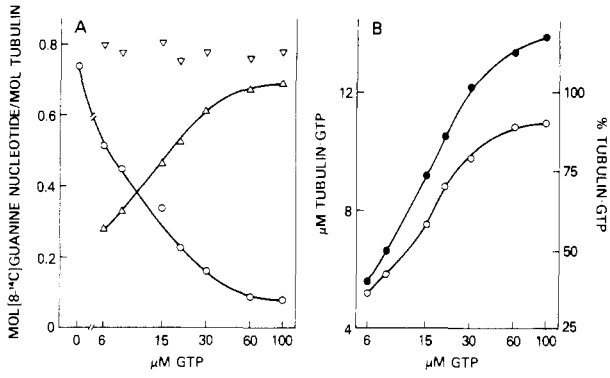

FIGURE 2: Effect of GTP concentration on the relative amounts of tubulin–GTP and tubulin–GDP in reaction mixtures. Each 0.43-mL reaction mixture contained 20 μM (2.0 mg/mL) tubulin, 0.1 M Mes (pH 7.0), 0.2 mM $MgCl_2$, and the indicated concentration of GTP. One set of reaction mixtures contained tubulin initially bearing [8-^{14}C]GDP in the exchangeable site and nonradiolabeled GTP, while a second set contained tubulin initially bearing nonradiolabeled GDP in the exchangeable site and [8-^{14}C]GTP. After 15 min at 0 °C, the samples were processed by centrifugal gel filtration as described in the text. In panel A, the data are expressed as moles of GTP (△), moles of GDP (○), and total moles of guanine nucleotide (▽) bound per mole of tubulin. In panel B, the data are expressed as micromolar tubulin–GTP (●) and as percent of nucleotide-bearing tubulin in the form of tubulin–GTP (○). The values for moles of GDP per mole of tubulin were obtained from the first set of reaction mixtures; the values for moles of GTP per mole of tubulin were obtained from the second set. The other values plotted in the figure were derived from these two measured quantities.

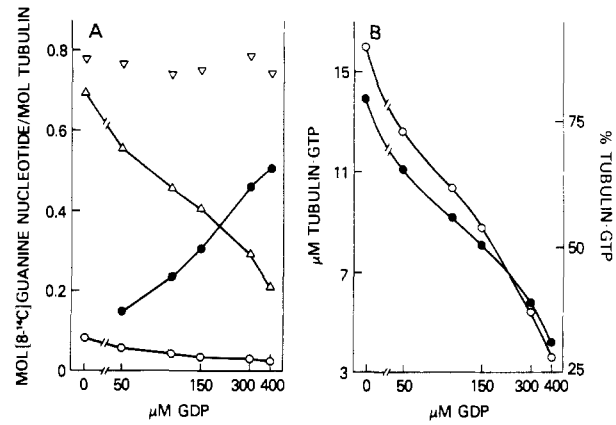

FIGURE 3: Effect of exogenously added GDP on the relative amounts of tubulin–GTP and tubulin–GDP in reaction mixtures. Each 0.43-mL reaction mixture contained 20 μM (2.0 mg/mL) tubulin, 0.1 M Mes (pH 7.0), 0.2 mM $MgCl_2$, 100 μM GTP, and the indicated concentration of GDP. One set of reaction mixtures contained tubulin initially bearing [8-^{14}C]GDP in the exchangeable site, nonradiolabled GDP, and nonradiolabeled GTP. A second set contained tubulin initially bearing nonradiolabeled GDP in the exchangeable site, [8-^{14}C]GDP, and nonradiolabeled GTP. A third set contained tubulin initially bearing nonradiolabeled GDP in the exchangeable site, nonradiolabeled GDP, and [8-^{14}C]GTP. After 15 min at 0 °C, the samples were processed by centrifugal gel filtration as described in the text. In panel A, the data are expressed as moles of guanine nucleotide bound per mole of tubulin, as follows: the symbols (○) represent moles of GDP initially bound in the exchangeable site retained by tubulin, obtained from the first set of reaction mixtures; the symbols (●) represent moles of exogenously added GDP bound by the tubulin, obtained from the second set; the symbols (△) represent moles of GTP bound by the tubulin, obtained from the third set; and the symbols (▽) represent total moles of guanine nucleotide bound by the tubulin, obtained by summing the results obtained from all three sets of reaction mixtures. In panel B, the data are expressed as micromolar tubulin–GTP (●) and as percent of nucleotide-bearing tubulin in the form of tubulin–GTP (○). These values were derived from those presented in panel A.

protein concentration was altered. The shortest lag phase was observed with 10 μM tubulin (+0.33 mg/mL MAPs) (curve 1), the fastest reaction rates with 10 and 20 μM tubulin (+0.33 and 0.67 mg/mL MAPs, respectively) (curves 1 and 2), and the most extensive reactions with 20 and 30 μM tubulin (+0.67 and 1.0 mg/mL MAPs, respectively) (curves 2 and 3). With 50 and 60 μM tubulin (+1.67 and 2.0 mg/mL MAPs, respectively) (curves 5 and 6), the turbidity tracings were virtually identical, but only with 50 μM tubulin (+1.67 mg/mL MAPs) was there a cold-reversible component to the final absorbance reading. Consequently, microtubule assembly was probably negligible at 60 μM tubulin (+2.0 mg/mL MAPs), particularly relative to the total amount of tubulin and MAPs in the reaction mixture.

Figure 2A presents the results of experiments in which the amounts of tubulin–GDP and tubulin–GTP in reaction mixtures were determined as a function of GTP concentration at 20 μM tubulin (comparable to the reaction condition used in the experiments of Figure 1A). The average total exchangeable nucleotide stoichiometry (relative to tubulin) in this series of experiments was 77%, not very different from the values obtained when the nucleotide content of the tubulin preparations was determined following denaturation of the protein (1.7 mol of total nucleotide/mol of tubulin). Not surprisingly, there is a steady decline in tubulin–GDP concentration and rise in tubulin–GTP concentration as the exogenous GTP concentration was increased, with the total exchangeable nucleotide essentially constant.

In Figure 2B, these data are expressed in two additional ways: the closed symbols indicate the concentration of tubulin–GTP present at each GTP concentration; the open symbols represent the percent of the tubulin bearing exchangeable nucleotide which is in the form of tubulin–GTP (i.e., tubulin without exchangeable nucleotide was excluded from the calculation). Turning to the GTP concentrations that define a polymerization threshold, 6 and 8 μM GTP, we obtained the following values for tubulin–GTP: 5.6 μM at 6 μM exogenous GTP (i.e., the free GTP concentration was thus 0.4 μM) and 6.6 μM at 8 μM exogenous GTP. The percent nucleotide-bearing tubulin in the form of tubulin–GTP was 36% and 42% at 6 and 8 μM exogenous GTP, respectively.

Figure 3A presents a similar series of experiments with 20 μM tubulin and 100 μM GTP (comparable to the reaction condition of Figure 1B) in which the effect of increasing concentrations of exogenous GDP on the amounts of tubulin–GDP and tubulin–GTP was examined. In this series of experiments, the average total exchangeable nucleotide stoichiometry was 76%. An increasing concentration of GDP, with the concentrations of GTP and tubulin held constant, resulted in a progressive reduction in the concentration of tubulin–GTP and a progressive rise in the total concentration of tubulin–GDP. It should be noted, moreover, that the higher the exogenous GDP concentration the lower the concentration of tubulin–GDP derived from GDP originally bound at the exchangeable site [symbols (○)], as would be predicted. These data, too, were expressed in terms of tubulin–GTP concentration (Figure 3B, closed symbols) and percent of nucleotide-bearing tubulin in the form of tubulin–GTP (Figure 3B, open symbols). The values for these parameters obtained for the polymerization threshold defined by GDP inhibition of assembly (400 and 300 μM, see Figure 1B) are 4.2 μM tubulin–GTP and 28% tubulin–GTP at 400 μM GDP, and 5.8 μM tubulin–GTP and 37% tubulin–GTP at 300 μM GDP.

One additional calculation of interest can be made from the data of Figures 2 and 3. Assuming that the 20–25% of the tubulin in the reaction mixtures which does not bind nucleotide does not participate in the nucleotide equilibria, we can set

Table II: Apparent Equilibrium Constant for the Exchange of GTP for GDP at the Exchangeable Site of Tubulin[a]

nucleotide(s) added	K_{eq}[b]
6 μM GTP	6.7
8 μM GTP	3.9
15 μM GTP	2.4
20 μM GTP	3.1
30 μM GTP	3.0
60 μM GTP	2.7
100 μM GTP	1.5
100 μM GTP + 50 μM GDP	1.9
100 μM GTP + 100 μM GDP	2.0
100 μM GTP + 150 μM GDP	2.1
100 μM GTP + 300 μM GDP	1.9
100 μM GTP + 400 μM GDP	1.7
	av 2.7

[a] Calculations were performed by using the average total nucleotide bound (7.62 pmol of nucleotide/μg of tubulin) from the experiments presented in Figures 2 and 3, together with the percent tubulin–GDP and the percent tubulin–GTP for each reaction condition (tubulin lacking exchangeable nucleotide was not included). The formal equation used in these calculations was the following: tubulin–GDP + GTP ⇄ tubulin–GTP + GDP. [b] Dimensionless constant.

up the following formal equation for the nucleotide exchange reaction:

$$\text{tubulin–GDP} + \text{GTP} \rightleftharpoons \text{tubulin–GTP} + \text{GDP}$$

Table II summarizes the equilibrium constants derived from the 12 distinct reaction conditions for which the calculation can be made from the data summarized in Figures 2 and 3. These values ranged from 1.5 to 6.7, with an average of 2.7 (a dimensionless constant). It thus appears that the affinity of tubulin for GTP [in 0.1 M Mes (pH 7.0)–0.2 mM $MgCl_2$] is about 3 times greater than its affinity for GDP, a conclusion not greatly different from that reached by several other groups, generally by more indirect methods (Arai et al., 1975; Carlier & Pantaloni, 1978; Zeeberg & Caplow, 1979; Fishback & Yarbrough, 1984).

Next we examined the effect of varying the tubulin concentration with the GTP concentration held constant at 15 μM (comparable to the reaction condition of Figure 1C) for its effect on the amounts of tubulin–GDP and tubulin–GTP in reaction mixtures (Figure 4A). These data, too, were expressed in terms of tubulin–GTP concentration (Figure 4B, closed symbols) and the percent of nucleotide-bearing tubulin in the form of tubulin–GTP (Figure 4B, open symbols). The average total exchangeable nucleotide stoichiometry in this series of experiments was 77%. As would be predicted, increasing the amount of tubulin in the reaction mixture resulted in a steady decline in the proportion of tubulin–GTP in the reaction mixture, with an offsetting rise in the proportion of tubulin–GDP. This undoubtedly accounts for the optimal assembly reactions observed at 10–20 μM tubulin (+MAPs) with 15 μM GTP (see Figure 1C). Nonetheless, there was a steady increase in the actual concentration of tubulin–GTP at least up to 40 μM tubulin (13.8 μM tubulin–GTP), and perhaps up to 50 μM tubulin (15.4 μM tubulin–GTP), although the latter value slightly exceeds the nominal concentration of exogenous GTP added to the reaction mixtures.

The polymerization threshold defined by 50 and 60 μM tubulin (+MAPs) yielded values, respectively, of 15.4 and 14.3 μM tubulin–GTP, and these differed little from the value of 13.8 μM tubulin–GTP obtained with 40 μM tubulin, a reaction condition with a more significant assembly reaction. The amounts of tubulin–GTP were substantially smaller at lower tubulin concentrations with brisker and more extensive polymerization reactions (5.2, 9.3, and 11.3 μM tubulin–GTP at, respectively, 10, 20, and 30 μM tubulin). It is rather the

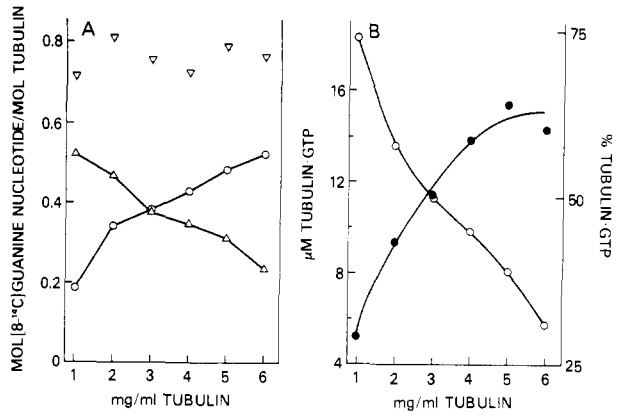

FIGURE 4: Effect of tubulin concentration on the relative amounts of tubulin–GTP and tubulin–GDP in reaction mixtures. Each 0.43-mL reaction mixture contained 0.1 M Mes (pH 7.0), 0.2 mM $MgCl_2$, 15 μM GTP, and the indicated concentration of tubulin. One set of reaction mixtures contained tubulin initially bearing [8-^{14}C]GDP in the exchangeable site and nonradiolabeled GTP, while a second set contained tubulin initially bearing nonradiolabeled GDP in the exchangeable site and [8-^{14}C]GTP. After 15 min at 0 °C, the samples were processed by centrifugal gel filtration as described in the text. In panel A, the data are expressed as moles of GTP (△), moles of GDP (○), and total moles of guanine nucleotide (▽) bound per mole of tubulin. In panel B, the data are expressed as micromolar tubulin–GTP (●) and percent of nucleotide-bearing tubulin in the form of tubulin–GTP (○). The values for moles of GDP per mole of tubulin were obtained from the first set of reaction mixtures; the values for moles of GTP per mole of tubulin were obtained from the second set. The other values plotted in the figure were derived from these two measured quantities.

decline in the percent of nucleotide-bearing tubulin as tubulin–GTP which is the significant value at the polymerization threshold observed at 50–60 μM tubulin with 15 μM GTP. This value is 31% at 60 μM tubulin and 39% at 50 μM tubulin (in contrast, it is 74% at 10 μM tubulin and 58% at 20 μM tubulin, the two protein concentrations associated with the most vigorous assembly reactions).

To summarize these findings, at 0.1 M Mes (pH 7.0)–0.2 mM Mg^{2+} polymerization thresholds, in terms of the percent of nucleotide-bearing tubulin, were observed at 36–42% tubulin–GTP (6 and 8 μM GTP with 20 μM tubulin), 28–37% tubulin–GTP (400 and 300 μM GDP with 20 μM tubulin and 100 μM GTP), and 31–39% tubulin–GTP (60 and 50 μM tubulin with 15 μM GTP). These values are quite similar, averaging about 35%. Thus, in this reaction condition, at least a third of the nucleotide bound in the exchangeable site must be GTP for microtubule assembly to occur.

The fourth reaction component, the Mg^{2+} concentration, which has significant effects on the proportion of GDP directly incorporated into microtubules, however, did not fall into this pattern. We had previously found that the lower the Mg^{2+} concentration, the higher the proportion of GDP directly incorporated into microtubules (Hamel et al., 1986b), and this was consistent with our finding that Mg^{2+} markedly enhanced the binding of GTP, but not GDP, to tubulin (Huang et al., 1985b).

We selected five exogenous Mg^{2+} concentrations to study in detail: 0, 0.2, 0.5, 2.0, and 4.0 mM.[5] First, we examined the assembly reaction with 20 μM tubulin (+0.67 mg/mL

[5] The basic reaction system consisting of 0.1 M Mes (pH 7.0), 2.0 mg/mL (20 μM) tubulin, and 0.67 mg/mL heat-treated MAPs was examined by atomic absorption spectroscopy for Mg^{2+} content, and a value of 25 μM was obtained. This includes the molar equivalent of Mg^{2+} tightly bound by tubulin (Olmsted & Borisy, 1975). The nonradiolabeled GTP preparation was also examined for Mg^{2+} content, and no significant Mg^{2+} was detected.

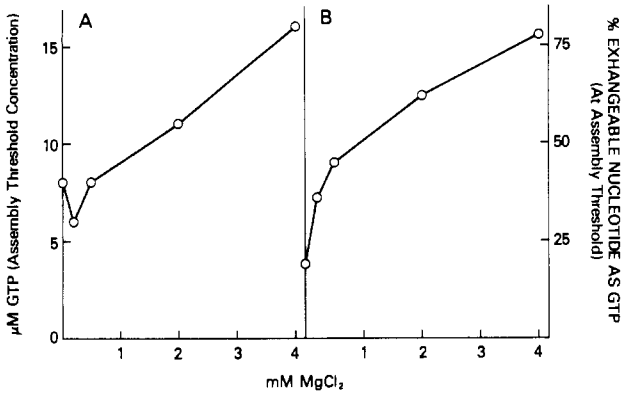

FIGURE 5: Effect of exogenous $MgCl_2$ on tubulin–nucleotide interactions. (A) Effect of exogenous $MgCl_2$ concentration on the minimum amount of GTP required for microtubule assembly. Each 0.25-mL reaction mixture contained 20 μM (2.0 mg/mL) tubulin, 0.67 mg/mL heat-treated MAPs, 0.1 M Mes (pH 7.0), the indicated concentration of exogenous $MgCl_2$, and varying amounts of GTP. Microtubule assembly was followed by turbidimetry (polymerization at 37 °C, depolymerization at 0 °C). The figure presents the highest concentration of GTP at each $MgCl_2$ concentration which never succeeded in supporting a significant microtubule assembly reaction (defined as a cold-reversible change in turbidity of greater than 0.01 A_{350} unit; see Figure 1A for a typical experiment at 0.2 mM exogenous $MgCl_2$). These values are termed "assembly threshold concentrations" of GTP. (B) Effect of exogenous $MgCl_2$ concentration on the proportion of nucleotide-bearing tubulin in the form of tubulin–GTP at assembly threshold concentrations of GTP. Each 0.43-mL reaction mixture contained 20 μM (2.0 mg/mL) tubulin, 0.1 M Mes (pH 7.0), the threshold GTP concentration (see panel A), and the indicated concentrations of exogenous $MgCl_2$. One set of reaction mixtures contained tubulin initially bearing [8-^{14}C]GDP in the exchangeable site and nonradiolabeled GTP (to yield the amount of tubulin–GDP in the reaction mixtures), while a second set contained tubulin initially bearing nonradiolabeled GDP in the exchangeable site and [8-^{14}C]GTP (to yield the amount of tubulin–GTP in the reaction mixtures). After 15 min at 0 °C, centrifugal gel filtration was performed as described in the text. The data are expressed as percent of nucleotide-bearing tubulin in the form of tubulin–GTP (tubulin without exchangeable nucleotide was not included in the calculation). The specific GTP concentrations examined were 8 μM without exogenous $MgCl_2$, 6 μM with 0.2 mM $MgCl_2$, 8 μM with 0.5 mM $MgCl_2$, 11 μM with 2 mM $MgCl_2$, and 16 μM with 4 mM $MgCl_2$.

Table III: Comparison of Relative Amounts of Tubulin–GDP in Reaction Mixtures and Tubulin–GDP Directly Incorporated into Microtubules

reaction components[a]			column A % GDP bound in exchangeable site[b]	column B % GDP directly incorporated into microtubules[c]	B/A[d]
tubulin (μM)	GTP (μM)	GDP (μM)			
10	15	0	26	14	0.54
20	15	0	42	25	0.60
30	15	0	50	32	0.64
20	20	0	30	19	0.63
20	30	0	21	13	0.62
20	60	0	11	6.9	0.63
20	100	0	10	5.5	0.55
20	100	50	27	15	0.56
20	100	100	38	22	0.58
20	100	150	46	28	0.61

[a] Reaction mixtures contained 0.1 M Mes (pH 7.0), 0.2 mM $MgCl_2$, and the indicated concentrations of tubulin, GTP, and, if indicated, GDP. In microtubule assembly experiments, reaction mixtures also contained heat-treated MAPs in a 1:3 weight ratio to the tubulin. Two or three (when exogenous GDP was added to the reaction) sets of reaction mixtures were used for each experimental condition: tubulin initially bearing [8-^{14}C]GDP in the exchangeable site + nonradiolabeled GTP (and GDP); tubulin initially bearing nonradiolabeled GDP in the exchangeable site + [8-^{14}C]GTP (and nonradiolabeled GDP); and, if required, tubulin initially bearing nonradiolabeled GDP in the exchangeable site, nonradiolabeled GTP, and [8-^{14}C]GDP. [b] Derived from data presented in Figures 2, 3, and 4. Only the nucleotide-bearing tubulin was used to calculate the percent GDP bound in the exchangeable site. [c] For each experimental condition, microtubule pellets were obtained [methodology described in detail in Hamel et al. (1986b)] from 0.25-mL reaction mixtures containing the components described above. Radiolabeled nucleotide originally added as GDP (either initially bound to the tubulin or as exogenous GDP) recovered in the pellet was taken to represent GDP directly incorporated into microtubules, while radiolabeled nucleotide originally added as GTP was taken to represent nucleotide which had been hydrolyzed to GDP in the course of assembly [see Hamel et al. (1986a)], which we term hydrolytic incorporation of GDP into microtubules. Only nucleotide-bearing tubulin which had entered the microtubule pellet was used to calculate the percent GDP directly incorporated into microtubules [i.e., % direct = 100[direct/(direct + hydrolytic)]]. [d] The values in column B divided by those in column A.

MAPs) at low GTP concentrations to find the minimum amount of the nucleotide required for polymerization, again defining the threshold reaction condition as one with a cold-reversible change in turbidity of less than 0.01 A_{350} unit (Figure 5A). Surprisingly, except for the reaction condition without exogenous $MgCl_2$, the threshold GTP concentration rose rather than fell as the Mg^{2+} concentration was increased. At the above five Mg^{2+} concentrations, no significant assembly occurred at, respectively, 8, 6, 8, 11, and 16 μM GTP. An almost parallel curve was observed in experiments in which a cold-reversible turbidity reading at the polymerization plateau of 0.05–0.10 A_{350} unit was obtained (12, 8, 10, 14, and 22 μM GTP at 0, 0.2, 0.5, 2.0, and 4.0 mM Mg^{2+}, respectively).

Equally striking was the steady increase in the percent of tubulin–GTP which was required for polymerization as the Mg^{2+} concentration rose. At the threshold GTP concentrations, tubulin–GTP rose from 19% (3.3 μM tubulin–GTP) in the absence of exogenous Mg^{2+} to 78% (13.4 μM tubulin–GTP) with 4 mM Mg^{2+} (Figure 5B). A similar rise in the proportion of tubulin–GTP was observed at the GTP concentrations in which a cold-reversible turbidity change of 0.05–0.10 A_{350} unit occurred: tubulin–GTP rose from 25% without exogenous Mg^{2+} (12 μM GTP) to 42% at 0.2 mM Mg^{2+} (8 μM GTP) to 52% at 0.5 mM Mg^{2+} (10 μM GTP) to 71% at 2.0 mM Mg^{2+} (14 μM GTP) to 85% at 4.0 mM Mg^{2+} (22 μM GTP). In addition, in agreement with our earlier observations (Huang et al., 1985b), at a constant GTP concentration, the proportion of tubulin binding GTP instead of GDP rose steadily as the Mg^{2+} concentration increased (data not presented).

To summarize these findings with Mg^{2+}, although at constant tubulin and GTP concentrations the percent of nucleotide-bearing tubulin in the form of tubulin–GTP increases as the Mg^{2+} concentration rises, the percent tubulin–GTP required for the initiation of microtubule assembly also increases. The reason for this apparent paradox is at present not clear.

Tubulin–GDP and Tubulin–GTP in Microtubule Elongation. The second aspect of the tubulin–GDP/tubulin–GTP equilibrium which we wished to study in some detail was the relationship between the amount of tubulin–GDP in a reaction mixture and the amount of GDP directly incorporated into microtubules. Specifically, we wished to determine whether the direct incorporation of GDP into microtubules was equal to the amount of tubulin–GDP in the reaction mixture, indicating that elongation with tubulin–GDP was as efficient as elongation with tubulin–GTP. Alternatively, less, or more, GDP might be directly incorporated into microtubules than predicted from the amount of tubulin–GDP in the reaction mixture, which would be consistent with less, or more, efficient elongation with tubulin–GDP than with tubulin–GTP.

Table III and Figure 6 summarize a series of experiments to answer this question. We restricted our analysis to reaction

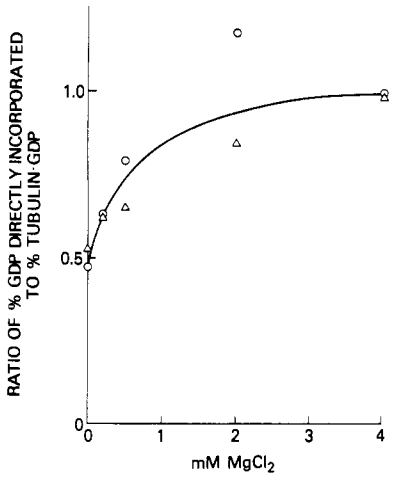

FIGURE 6: Effect of $MgCl_2$ concentration on the efficiency of direct incorporation of tubulin–GDP into microtubules. Two series of experiments were performed, at 30 μM (Δ) and 60 μM (O) GTP, at each of the indicated concentrations of exogenously added $MgCl_2$. The percent of direct incorporation of GDP was determined as described in Table III, in reaction mixtures incubated for 15 min at 37 °C and containing 20 μM (2.0 mg/mL) tubulin, 0.67 mg/mL heat-treated MAPs, 0.1 M Mes (pH 7.0), and the indicated $MgCl_2$ and GTP concentratons. The percent tubulin–GDP was determined as described in Figures 2–5, in reaction mixtures incubated for 15 min at 0 °C and containing 20 μM (2.0 mg/mL) tubulin, 0.1 M Mes (pH 7.0), and the indicated $MgCl_2$ and GTP concentrations. Each data point, representing the ratio of percent direct GDP incorporation into microtubules to the percent tubulin–GDP in an analogous reaction mixture, involved determination of four quantities: tubulin–GTP in the reaction mixture; hydrolytic incorporation of GDP into microtubules (i.e., incorporation of tubulin–GTP into microtubules); tubulin–GDP in the reaction mixture; and direct incorporation of GDP into microtubules (i.e., incorporation of tubulin–GDP into microtubules). The first two values were obtained by using tubulin initially bearing nonradiolabeled GDP in the exchangeable site + [8-^{14}C]GTP; the last two values were obtained by using tubulin initially bearing [8-^{14}C]GDP in the exchangeable site + nonradiolabeled GTP.

conditions with fairly extensive assembly reactions (a cold-reversible turbidity reading of at least 0.10 A_{350} unit). Table III presents data obtained in experiments with 0.2 mM exogenous Mg^{2+}. Column A in the table summarizes data derived from experiments presented earlier in Figures 2–4, but in column A the percent of nucleotide-bearing tubulin in the form of tubulin–GDP (rather than tubulin–GTP) is presented.

To obtain the data presented in Table III, column B, microtubule pellets were prepared as described in detail elsewhere (Hamel et al., 1986b) from equivalent reaction mixtures containing either nonradiolabeled GTP and tubulin bearing [8-^{14}C]GDP in the exchangeable site (to yield data on the direct incorporation of GDP into microtubules) or [8-^{14}C]GTP and tubulin bearing nonradiolabeled GDP in the exchangeable site [to yield data on the hydrolytic incorporation of GDP into microtubules, for virtually all the radiolabeled nucleotide in the pellet is in the form of GDP; see Hamel et al. (1986a)]. The ratios of direct GDP incorporation to total GDP incorporation (direct + hydrolytic) are presented in column B. (For the 10 experimental conditions described in Table III, the average value for moles of total exchangeable nucleotide per mole of tubulin in the pellet was 0.84.)

Finally, the values from column B were divided by those in column A to yield ratios representing a measure of the efficiency with which tubulin–GDP was incorporated into microtubules relative to its concentration in the original reaction mixtures (prior to the onset of polymerization).

The series of experiments in which the concentrations of GTP, GDP, and tubulin were varied at an exogenous Mg^{2+} concentration of 0.2 mM produced ratios within a limited range (0.54–0.64, average 0.60). It thus appears that at 0.2 mM Mg^{2+}, regardless of nucleotide or tubulin concentrations, tubulin–GDP is incorporated into microtubules about 60% as efficiently as would be predicted on the basis of its concentration in the reaction mixtures.

The effect of varying the Mg^{2+} concentration, however, was again quite different (Figure 6). Two series of experiments were performed at 30 and 60 μM GTP. The ratio of tubulin–GDP directly incorporated into microtubules relative to its concentration in the reaction mixture was lowest in the absence of exogenous Mg^{2+} (about 0.5) and progressively rose as the Mg^{2+} concentration was increased. At the highest Mg^{2+} concentrations, in fact, these ratios were about 1, suggesting that tubulin–GDP was incorporated into microtubules as efficiently as would be predicted on the basis of its concentration in the reaction mixture. Consequently, at the highest Mg^{2+} concentrations, tubulin–GDP and tubulin–GTP are incorporated into microtubules with approximately equal efficiency.

Discussion

In a recent study, we showed that tubulin–GDP is directly incorporated into microtubules throughout elongation in a relatively constant proportion, with the balance between direct incorporation of tubulin–GDP and incorporation of tubulin–GTP (with GTP hydrolysis) dependent on precise reaction conditions (Hamel et al., 1986b). Direct tubulin–GDP incorporation is favored by changes in the reaction mixture which increase its concentration: low Mg^{2+} concentrations (Huang et al., 1985b), adding relatively low concentrations of GDP (but not enough to completely inhibit microtubule assembly) to the reaction, reducing the GTP concentration with the tubulin concentration held constant, or raising the tubulin concentration with the GTP concentration held constant. [Changes in the concentration of MAPs had only minimal effects on the direct incorporation of GDP into microtubules (Hamel et al., 1986b).]

In the studies presented here, we have carefully examined the effects of these reaction components on the relative amounts of tubulin–GDP and tubulin–GTP in reaction mixtures, exploiting the tenacious binding of guanine nucleotides to tubulin at high reaction pH values (Zeeberg & Caplow, 1979; Hamel et al., 1986a) and the virtually complete inhibition of nucleotide binding and release at the exchangeable site by the antimitotic drug maytansine (Huang et al., 1985a) to obtain values which are probably close to those that exist at equilibrium. Although our studies were not exhaustive, we have thus far noted no significant effect of either temperature or heat-treated MAPs on the tubulin–GDP/tubulin–GTP equilibrium.[6,7]

[6] As noted above, this would seem to be in disagreement with the report of Caplow and Zeeberg (1980) that only limited nucleotide exchange occurred in ring oligomers, but this may be related to our use of only a subset of the MAPs.

[7] This is perhaps not all that surprising, since tubulin without MAPs at 0 °C binds GTP quite avidly. Included in the reaction conditions we examined at 0.2 mM exogenous Mg^{2+} were seven conditions in which GTP was added at a concentration substoichiometric to that of tubulin (20 μM tubulin with 6, 8, and 15 μM GTP; and 30, 40, 50 and 60 μM tubulin with 15 μM GTP). In these seven experiments, the tubulin bound an average of 86% of the added GTP (range, 61–100%). Thus, MAPs and increased temperatures had little possibility of greatly enhancing the affinity of GTP for tubulin.

Our goal was 2-fold. First, we wanted to determine whether differing reaction conditions which represented "thresholds" for microtubule assembly (defined as existing when a relatively small change in a single reaction component leads to a partial microtubule assembly reaction) would be similar in their tubulin–GTP:tubulin–GDP ratios, since GTP was an absolute requirement for polymerization in the experiments presented here. Second, we wanted to determine whether tubulin–GDP was incorporated directly into microtubules as efficiently as tubulin–GTP. For both questions, we found that the effect of varying the Mg^{2+} concentration produced a result distinct from that obtained by varying the GTP, GDP, and tubulin concentrations.

When the latter three parameters were varied at 0.2 mM exogenous Mg^{2+} (but 0.225 mM total Mg^{2+} if the cation contained in the reaction components is included), we found relatively little variation in the tubulin–GTP to tubulin–GDP ratios observed at polymerization thresholds, and these averaged just over 1:2 (tubulin–GTP was about 35% of the nucleotide-bearing tubulin at polymerization thresholds in 0.2 mM exogenous Mg^{2+}). It must be stressed that it was not the actual molar concentration of tubulin–GTP but rather its proportion of the total tubulin which defined whether there would be initiation of microtubule assembly. At the 15 μM GTP used in the studies of Figures 1C and 4, microtubule assembly was clearly optimal at 10–20 μM tubulin (+0.33–0.67 mg/mL MAPs), even though the concentration of tubulin–GTP was 1.5–3-fold greater at 40–60 μM tubulin. In this series of experiments, the percent tubulin–GTP fell steadily from 74% at 10 μM tubulin to 31% at 60 μM tubulin.

Similarly, when direct incorporation of GDP into microtubules was compared to the proportion of tubulin–GDP in reaction mixtures at 0.2 mM exogenous Mg^{2+} at a wide range of tubulin, GTP, and GDP concentrations, relatively homogeneous results were obtained: direct incorporation was about 60% (range 54–64%) of the value predicted if elongation by tubulin–GDP was equal to its proportional concentration in the reaction mixtures. Thus, not surprisingly, at 0.2 mM exogenous Mg^{2+} elongation with tubulin–GDP was less efficient than elongation with tubulin–GTP. Nonetheless, elongation with tubulin–GDP relative to elongation with tubulin–GTP was significantly greater than might be predicted from the 2.7:1 relative binding of GTP and GDP to tubulin (obtained from the average equilibrium constant for exchange of GTP for GDP, see Table II); i.e., the relative affinity of tubulin–GDP for microtubules appears to be greater than the relative affinity of GDP for tubulin.

Mg^{2+} has multiple, seemingly paradoxical effects on the microtubule assembly system under study here. Earlier work (Huang et al., 1985b) established that binding of GTP to the exchangeable site of tubulin is markedly enhanced by Mg^{2+}, rising as the Mg^{2+} concentration increases, while GDP binding to the exchangeable site is not dependent on the Mg^{2+} concentration and may even be somewhat inhibited at higher Mg^{2+} concentrations.[8] In the present studies, we initially determined the minimum GTP concentration required for microtubule assembly at five different Mg^{2+} concentrations. We found that the lowest GTP concentration (8 μM) for significant polymerization occurred with 0.2 mM exogenous Mg^{2+} and, although slightly higher in the absence of exogenous Mg^{2+}, rose steadily as the Mg^{2+} concentration was raised from 0.2 to 4.0 mM.

Since the amount of GTP bound at the exchangeable site rises with the exogenous Mg^{2+} concentration (Huang et al., 1985b), the proportion of nucleotide-bearing tubulin in the form of tubulin–GTP at polymerization thresholds[9] steadily increases with the exogenous Mg^{2+} concentration. Thus, in the absence of exogenous Mg^{2+}, the threshold proportion of tubulin–GTP was between 19% and 25% (the values obtained at 8 and 12 μM GTP), while at 0.5 mM exogenous Mg^{2+} the threshold proportion was between 40% and 52% (the values obtained at 8 and 10 μM GTP) and at 4.0 mM exogenous Mg^{2+} the threshold proportion was between 78% and 85% (the values obtained at 16 and 22 μM GTP).

Examination of the effect of Mg^{2+} on the efficiency of incorporation of tubulin–GDP into microtubules produced equally unexpected results. At the lower Mg^{2+} concentrations, the proportion of tubulin–GDP in the reaction mixture was higher, but tubulin–GDP was less efficiently incorporated into microtubules than was the case at the higher Mg^{2+} concentrations. Even though at the highest Mg^{2+} concentrations examined (2.0 and 4.0 mM) there was relatively little tubulin–GDP in the reaction mixtures, what remained was incorporated into microtubules with high efficiency, comparable to the efficiency with which tubulin–GTP entered microtubules.

To summarize these observations with Mg^{2+}: (1) as the Mg^{2+} concentration increases, the binding of GTP to tubulin becomes progressively more extensive; but (2) at the same time the proportion of nucleotide-bearing tubulin in the form of tubulin–GTP required for the initiation of microtubule assembly becomes progressively higher; and (3) the direct incorporation of residual tubulin–GDP into microtubules becomes more efficient as its proportion of the nucleotide-bearing tubulin declines.

There are thus multiple effects of Mg^{2+} on tubulin polymerization in addition to its direct stimulation of the binding of GTP to the protein. The findings that Mg^{2+}, on the one hand, increases the proportion of tubulin–GTP required for the initiation of microtubule assembly and, on the other, enhances the direct incorporation of tubulin–GDP into microtubules during elongation (or, alternatively, reduces the incorporation of tubulin–GTP) demonstrate that the cation has significant effects on tubulin–tubulin interactions, perhaps modulated by the MAPs. It would appear, in fact, that beyond a relatively low concentration of Mg^{2+} (of the order of 0.2 mM) probably required for adequate binding of GTP to the exchangeable site, Mg^{2+} interferes with specific interactions of tubulin–GTP required in both initiation and elongation.[10]

It is tempting, however, to explain the effects of Mg^{2+} described here in terms of formation of tubulin–MAPs oligomers that are required for microtubule assembly. Many workers have proposed essential roles for such oligomers in both the initiation and elongation phases of the reaction [for example, see Voter and Erickson (1984), Manser and Bayley (1985),

[8] Equilibrium constants for the exchange of GTP for GDP at the other four exogenous Mg^{2+} concentrations were also calculated, although the data were not as extensive as those obtained at 0.2 mM exogenous Mg^{2+}. The results (dimensionless constants) were as follows: 0.2 in the absence of exogenous Mg^{2+}; 5.0 with 0.5 mM Mg^{2+}; 10.8 with 2.0 mM Mg^{2+}; and 10.6 with 4.0 mM Mg^{2+}. Thus, in the absence of exogenous Mg^{2+}, the affinity of GTP for tubulin is only one-fifth that of GDP, but with added Mg^{2+}, the affinities of the two nucleotides reverse, with GTP binding 11 times as tightly as GDP at the higher Mg^{2+} concentrations.

[9] Defined in terms of the GTP concentrations at each Mg^{2+} concentration that produced cold-reversible turbidity readings of less than 0.01 A_{350} unit, on the one hand, and of 0.05–0.10 A_{350} unit, on the other.

[10] This may represent a modulating effect of the MAPs on tubulin–tubulin and tubulin–nucleotide interactions, since Mg^{2+} clearly enhances glycerol-dependent microtubule assembly from purified tubulin (Lee & Timasheff, 1975).

Howard and Timasheff (1986), and Spann et al. (1987)].[11] Although it is now generally agreed that the most morphologically distinct oligomers, single and double rings, are not direct participants in microtubule assembly (Howard & Timasheff, 1986; Spann et al., 1987), it is the recent report of Howard and Timasheff (1986) on the effects of Mg^{2+} and GDP on ring formation that forms the basis of our speculations. These workers found that ring formation by purified tubulin was markedly enhanced by higher Mg^{2+} concentrations (about 10 mM) and by GDP relative to GTP.

In extending the findings of Howard and Timasheff (1986) to our observations, we will make three assumptions: first, that the stabilization of tubulin rings by GDP and Mg^{2+} applies equally to other, thus far poorly defined, oligomers; second, that addition of MAPs to the reaction mixture shifts the stabilization of oligomers by Mg^{2+} to lower cation concentrations, for our "high" Mg^{2+} concentrations of 2 and 4 mM did not favor ring formation from purified tubulin under the conditions studied by Howard and Timasheff (1986); third, that MAPs favor tubulin–GTP oligomers, as opposed to tubulin–GDP oligomers, more than is the case in their absence. It is, moreover, likely, under the reaction conditions we have studied in greatest detail, that mixed oligomers containing both tubulin–GTP and tubulin–GDP would form.

With these assumptions, we can conclude that higher Mg^{2+} concentrations would tend to drive tubulin–GDP into oligomeric structures. Sufficient tubulin–GDP (perhaps as little as a single subunit) in an initiating oligomer [postulated by Voter and Erickson (1984) to be a heptamer] would poison nucleation, resulting in an ever higher GTP concentration required for assembly as the Mg^{2+} concentration increases. On the other hand, since GTP is not required for elongation (Carlier & Pantaloni, 1978; Karr et al., 1979; Zackroff et al., 1980), oligomers enriched in tubulin–GDP would readily participate in microtubule elongation, accounting for the efficient direct incorporation of GDP into microtubules at the higher Mg^{2+} concentrations.

We have made a preliminary search for such oligomers. First, we have examined reaction mixtures containing tubulin + MAPs at a range of Mg^{2+} concentrations in the electron microscope. While we have observed single and double rings under all conditions evaluated (even in the absence of exogenous Mg^{2+}), no other morphologically distinct structures were visualized. (Our studies to date have not permitted quantitation of ring formation vis-ã-vis Mg^{2+} concentration, but, qualitatively, differences are not remarkable.) We have also evaluated effects of temperature (in the absence of GTP), Mg^{2+}, and nucleotides on the turbidity of solutions of tubulin + MAPs, although changes in turbidity provide no specific morphological information (rings, other oligomers, and aggregates would all increase the turbidity of reaction mixtures). No significant changes were observed as a function of temperature or upon the addition of GDP, but Mg^{2+} caused small increases and GTP caused small decreases in turbidity. These preliminary observations thus suggest that reaction components can alter the aggregation state of tubulin + MAPs. The relationship of such aggregation changes, however, to the varying participation of tubulin–GDP and tubulin–GTP in microtubule assembly remains to be established.

Registry No. Mg, 7439-95-4.

[11] Manser and Bayley (1985), in their report on the direct incorporation of tubulin–GDP into microtubules during polymerization supported by guanosine 5′-(β,γ-methylenetriphosphate), postulated that the direct incorporation occurred during the late phase of the reaction, and they argued that the mechanism involved polymerization of oligomers partially composed of tubulin–GDP subunits. We find, however, that direct incorporation of tubulin–GDP into microtubules occurs throughout polymerization in a relatively constant proportion, not just late in the reaction (Hamel et al., 1986b).

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Calcium Channel Activity in a Purified Dihydropyridine–Receptor Preparation of Skeletal Muscle†

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ABSTRACT: A purified dihydropyridine–receptor complex (DHPR) of skeletal muscle consisting of a major polypeptide of M_r 150K under reducing conditions induces divalent cation selective channels when incorporated into planar lipid bilayers. Channels were inserted into preformed planar bilayers by two techniques: (i) direct dilution of detergent-solubilized DHPR into the aqueous chambers adjacent to the bilayer membrane or (ii) reconstitution of DHPR into phospholipid vesicles followed by fusion of the preformed vesicles to the planar bilayer membrane. Unlike native membrane preparations of t-tubules, which only have one major Ca channel type of slope conductance of 12 pS in symmetrical 100 mM Ba, the purified DHPR complex induced at least two channel types with conductances of 12–14 and 22 pS. Some recordings suggest that these two channels are statistically coupled in time, i.e., that they may correspond to substates of the same DHPR channel. Activity was found to occur spontaneously in the absence of the Ca channel agonist Bay k 8644. The 12–14-pS channel from DHPR exhibits voltage-dependent kinetics, is highly selective for barium ions, and was inhibited by micromolar nitrendipine. The 12–14-pS DHPR channel appears to be identical with functional Ca channels previously described in native t-tubules.

1,4-Dihydropyridines (DHP), phenylalkylamines, and benzothiazepines are a diverse group of compounds that can increase or decrease Ca channel activity, by inducing long-term opening or closing of Ca channels in several tissues (Schramm et al., 1983; Janis et al., 1984; Schwartz & Triggle, 1984; Brown et al., 1984; Hess et al., 1984; Kokubun & Reuter, 1984; Schwartz et al., 1985; Affolter & Coronado, 1985; Rosenberg et al., 1986; Caffrey et al., 1986; Vaghy et al., 1987a). The finding that transverse tubular membranes of skeletal muscle contain a large number of receptors that bind 1,4-dihydropyridines with high affinity (DHPR) has led to the solubilization and purification of the DHPR complex. A large peptide (M_r 135K–170K) appears to be a major subunit though many discrepancies exist about the presence of other possible subunits (Curtis & Catterall, 1984; Ferry et al., 1984; Galizzi et al., 1986; Flockerzi et al., 1986; Nakayama et al., 1986a,b, 1987; Cooper et al., 1987; Leung et al., 1987).

Skeletal muscle Ca channels, in vivo and in planar bilayers, are modulated by dihydropyridine agonists and antagonists (Charandini & Stefani, 1983; Schwartz et al., 1985; Affolter & Coronado, 1985). Thus, it has been of interest to investigate whether the purified DHPR can induce Ca flux or single-channel currents in reconstituted systems. Curtis and Catterall (1986) showed that a small fraction of DHPR promotes Ca flux in liposomes that can be increased by the dihydropyridine agonist Bay k 8644 and inhibited by several antagonists. Recordings of Ca single-channel activity present in DHPR has been recently described (Flockerzi et al., 1986). In this paper we show for the first time that a highly purified preparation of DHPR (Nakayama et al., 1986a,b, 1987) induces, among others, a 12–14-pS voltage-dependent, dihydropyridine-sensitive channel that is similar, on the basis of channel conductance, divalent ion selectivity, and drug sensitivity, to those identified in native t-tubule vesicles as bona fide DHP-sensitive Ca channels (Affolter & Coronado, 1985, 1986; Coronado & Affolter, 1986; Coronado & Smith, 1987). However, it is unclear whether Ca channels copurify with DHPR or DHPR per se constitutes a Ca channel. A preliminary report has been presented elsewhere (McKenna et al., 1987).

Materials and Methods

Purification of DHPR. t-Tubule membranes were prepared from rabbit skeletal fast (white) muscle as described by Nakayama et al. (1987). Briefly, these procedures employ freeze-thawed muscle and a French press treatment. Purification of DHPR from t-tubule membranes was performed as described by Curtis and Catterall (1984) and Nakayama et al. (1987), with minor modifications. t-Tubule membranes were not prelabeled with [^{3}H]-(+)-PN200-110 prior to solubilization with digitonin. Initial studies using prelabeled DHPR, as well as postlabeling experiments on each purifi-

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