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Nucleotide Specificity in Microtubule Assembly in Vitro†

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ABSTRACT: A procedure is described for removing most of the GDP bound at the exchangeable GTP binding site (E site) of tubulin. Microtubule protein containing substoichiometric amounts of GDP at the E site is found to polymerize in response to: (a) two nonhydrolyzable ATP analogues, adenylyl imidodiphosphate (AMP-PNP) and adenylyl β,γ-methylenediphosphonate (AMP-PCP); and (b) substoichiometric levels of GTP or dGTP. The results are interpreted as suggesting that: (1) when GDP is removed from tubulin, the E site shows broad specificity for nucleoside triphosphates; (2) microtubule assembly can be induced by the binding of substoichiometric amounts of nucleoside triphosphate to the E site.

Tubulin, the 110 000 molecular weight subunit of microtubules (Shelanski & Taylor, 1967; Weisenberg, 1972), binds 2 mol of GTP, one at an exchangeable or E site and one at a nonexchangeable or N site (Weisenberg et al., 1968). Exchangeably bound GTP is readily displaced by GTP or GDP in the medium, and the K_{diss} for GTP binding at the E site has been estimated at 10^{-6} to 10^{-7} mol L^{-1} (Jacobs & Caplow, 1976; Levi et al., 1974). At the N site no exchange of GTP has been detected in vitro; however, the release of N-site GTP on denaturation of the tubulin molecule indicates that it is noncovalently bound to tublin.

Recent work in several laboratories has led to an increased understanding of nucleotide requirements in microtubule assembly in vitro (Arai & Kaziro, 1976; Kobayashi & Simizu, 1976; Penningroth et al., 1976; Weisenberg et al., 1976; Penningroth & Kirschner, 1977). Two nonhydrolyzable GTP analogues, guanylyl β,γ-methylenediphosphonate (GMP-PCP) and guanylyl imidodiphosphate (GMP-PNP), as well as GTP have been found to induce microtubule assembly by binding to the E site. Other nucleoside triphosphates such as ATP, UTP, and CTP appear to act indirectly by phosphorylating exchangeably bound GDP in situ at the E site by means of a nucleoside diphosphate kinase (NDP-kinase) activity, which is not intrinsic to tubulin, and binding of the resulting GTP at the E site then induces microtubule assembly. GTP at the N site has been shown not to turn over during the polymerization reaction in vitro.

In previous examinations of the nucleotide specificity of the E site (Jacobs et al., 1974; Arai et al., 1975; Penningroth & Kirschner, 1977), no nucleotides other than GTP, GDP, GMP-PCP, and GMP-PNP were found to interact demonstrably with the E site, suggesting that the E-site may be absolutely specific for guanosine nucleotides. However, an absence of binding of weak nucleotide ligands to tubulin could be due to inhibition by GDP, which is present in most tubulin preparations. As a strong ligand, GDP could block the binding of weak ligands to the E site. GDP in the medium has been shown to inhibit microtubule polymerization with GMP-PCP and GMP-PNP (Penningroth et al., 1976). In this paper a method is described which removes approximately 60% of the GDP from the E site of tubulin. When the concentration of GDP at the E site is reduced, two weakly binding, nonhydrolyzable ATP analogues, adenylyl imidodiphosphate (AMP-PNP) and adenylyl β,γ-methylenediphosphonate (AMP-PCP) will induce microtubule assembly comparable in rate and extent to the polymerization reaction observed with GTP. Polymerization is also induced by weakly binding, hydrolyzable nucleoside triphosphates such as UTP and ATP, suggesting that the E site of tubulin possesses broad specificity for nucleoside triphosphates in the microtubule polymerization reaction. In addition, microtubule polymerization is shown to be promoted by substoichiometric concentrations of GTP and dGTP. The results are interpreted as suggesting that nucleoside triphosphates and GDP may act allosterically as antagonists in the microtubule polymerization reaction.

Materials and Methods

Preparation of Microtubule Protein. Microtubule protein was purified from porcine brain by alternate cycles of polymerization/depolymerization according to the procedure of Shelanski et al. (1973) as modified by Weingarten et al. (1974) and stored in purification buffer (0.1 M Mes, 0.5 mM $MgCl_2$, 1.0 mM β-mercaptoethanol, 2.0 mM EGTA, 0.1 mM EDTA (pH 6.4) with NaOH) containing 8 M glycerol plus 1 mM GTP at −20 °C. Protein was used within 4 weeks of purification. Prior to each experiment, microtubule protein was diluted 1:1 with purification buffer and repolymerized with 0.1 mM GTP for 30 min at 37 °C. Microtubules were centrifuged at 180 000*g* for 35 min at 25 °C in a Ti 50 fixed angle rotor (Beckman) using 10-mL polycarbonate tubes. The supernatant was discarded, and the surface of the pellet and the walls of the centrifuge tube were washed with a 1.0-mL aliquot of purification buffer to ensure the complete removal of the superna-

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tant. The microtubule pellet was resuspended in purification buffer at a concentration of approximately 3–4 mg/mL and depolymerized for 20 min at 4 °C. Acid-washed charcoal was mixed with the depolymerized microtubule protein at a concentration of 5 mg of charcoal per mL of protein solution, and the charcoal was removed by centrifugation at 12 000*g* for 5 min at 4 °C in a Sorvall SS 34 rotor. The charcoal treatment was then repeated. The protein prepared in this manner is referred to as GDP-tubulin.

Removal of GDP from the E Site. GDP-tubulin (3–4 mg/mL) was centrifuged for 15 min at 180 000*g* at 4 °C to remove large aggregates of denatured tubulin. The pellet was discarded, and the supernatant was repolymerized with 1.5 mM GMP-PCP for 30 min at 37 °C. Three to four milliliters of this microtubule suspension were pelleted through a 3-mL cushion of nucleotide-free purification buffer containing 4 M glycerol at 180 000*g* for 25 min at 25 °C in a Ti 50 fixed angle rotor (Beckman). The supernatant was carefully removed and the pellet and walls of the tube were washed two times with 1-mL aliquots of purification buffer without disturbing the pellet. The pellet was then resuspended in 1.5 to 2.0 mL of purification buffer and depolymerized for 20 min at 4 °C. The solution was made up to 0.75 M in NaCl and centrifuged for 15 min at 180 000*g* at 25 °C to pellet aggregates of denatured tubulin (Weingarten et al., 1974). The pellet was discarded and the supernatant was desalted into purification buffer at 4 °C on a polyacrylamide P-10 column, 100–200 mesh (Bio-Gel), sample to bed volume ratio approximately 5.0. The protein eluted from the column at a concentration of approximately 3 mg/mL and was tested empirically for removal of GDP by assaying turbidimetrically for microtubule assembly with a nonhydrolyzable ATP analogue. This protein, which is referred to as GDP/free tubulin, was used within 2 h of preparation.

Nucleotides. AMP-PCP and GMP-PCP were purchased from Boehringer Mannheim Corp., and AMP-PNP was obtained from Sigma Chemical Co. All other nucleotides were from Sigma. All nucleoside triphosphates used in this study except AMP-PNP were found to be 98–100% pure by thin-layer chromatography, the only detectable impurities being trace amounts of nucleoside diphosphate. AMP-PNP consisted of three components, the major one comprising approximately 70% of the total material. Since preparations of GMP-PNP have also been found to contain two impurities, tentatively identified as GMP-PN and GMP-PNH_2P (Penningroth & Kirschner, 1977; Penningroth, 1977), the impurities in the AMP-PNP preparation may have been AMP-PN and AMP-PNH_2P. All nucleotides were used without further purification.

Nucleotide Concentration. Molar extinction coefficients and spectral ratios were obtained from Cohn (1957). Concentrations of tubulin-associated nucleotides were determined from absorbance measurements at 260 nm following removal of protein by precipitation with 5% perchloric acid.

Polymerization Kinetics. The rate of microtubule assembly was followed turbidimetrically at 380 nm in a 1-cm pathlength quartz cuvette on a Beckman Acta C II spectrophotometer equipped with a thermostated cuvette holder and an Esterline Angus recorder. The occurrence of microtubule assembly was established on the basis of a typical S-shaped turbidimetric polymerization curve; a slow (less than 0.02 OD mg^{-1} mL^{-1} h^{-1}) linear increase in turbidity was symptomatic of nonmicrotubule aggregation as judged by electron microscopy. Questionable polymerization curves were verified by electron microscopy. Aggregated protein showed no discernible structures, while microtubules had normal morphology.

Other Methods. One-dimensional ascending chromatography was performed on PEI (polyethylenimine) cellulose flexible thin-layer sheets (Bakerflex). Chromatograms were developed at ambient temperature in 1.2 M LiCl according to the procedures of Randerath (1966) for a distance of approximately 13 cm from the origin. The preparation of tubulin-associated nucleotides for thin-layer chromatography was performed as described previously (Penningroth et al., 1976). Relative spot intensities were estimated visually under ultraviolet light.

Quantitative electron microscopy was performed on a JEOL 100C electron microscope using the spray-drop procedure of Kirschner et al. (1975).

Protein concentration was determined by the method of Lowry et al. (1951) using serum albumin as a standard.

Microtubule assembly was induced with a GTP-generating system by incubating microtubule protein with 60 μg/mL pyruvate kinase (Boehringer Mannheim Corp.) plus 1 mM phosphoenolpyruvate at 37 °C, as described by Kobayashi & Simizu (1976).

Results

Removal of GDP from the E Site. GDP is bound at the E site of tubulin with high affinity, and consequently the level of E site GDP is not reduced significantly by gel filtration or by extensive dialysis (Penningroth & Kirschner, unpublished results). To remove GDP, it was therefore necessary to displace it from tubulin by competitive binding of GMP-PCP to the E site. GMP-PCP as a relatively weak ligand (Penningroth & Kirschner, 1977) can then be removed by gel filtration. Following this procedure, the nucleotide: tubulin stoichiometry typically was reduced from 1.4:1.0 in GDP-tubulin to 1.0:1.0 in GDP/free tubulin. When nucleotides associated with GDP-tubulin and GDP/free tubulin were analyzed by thin-layer chromatography and a visual estimate of the concentration was made, the concentration of GDP was found to be reduced 60 to 80% in GDP/free tubulin. The GTP concentrations were approximately the same in both samples. No GMP-PCP was observed in the GDP/free tubulin, confirming that it was removed by the gel filtration step.

GDP-tubulin containing 1.4 mol of nucleotide/mol of tubulin polymerized on addition of ATP, but no polymerization was observed with two nonhydrolyzable ATP analogues, AMP-PNP or AMP-PCP. These results agree with earlier findings which had been interpreted in terms of a mechanism of nucleotide action in which ATP acts by phosphorylating exchangeably bound GDP to GTP (Kobayashi & Simizu, 1976; Weisenberg et al., 1976; Penningroth & Kirschner, 1977). In GDP/free tubulin, microtubule assembly could be promoted with either AMP-PCP or AMP-PNP (Figure 1). Microtubules of normal morphology were identified in the electron microscope. The initial rate of microtubule assembly with millimolar concentrations of AMP-PNP and AMP-PCP was found to vary up to a factor of three with different preparations of GDP/free tubulin, probably due to variability in the efficiency of GDP removal by our procedure. In the experiment in Figure 1, AMP-PNP induced assembly to about 85% of the level of GTP within 23 min, indicating that the extent of assembly stimulated by AMP-PNP is comparable to levels observed with GTP, GMP-PCP (Penningroth et al., 1976), and GMP-PNP (Weisenberg & Deery, 1976). Microtubule assembly with AMP-PNP was inhibited when GDP was added back to GDP/free tubulin (Figure 2). Figure 2 shows further that while 0.1 mM GDP, which binds to the E site, completely inhibited AMP-PNP-induced polymerization, 0.1 mM ADP, which binds weakly or not at all to the E site,

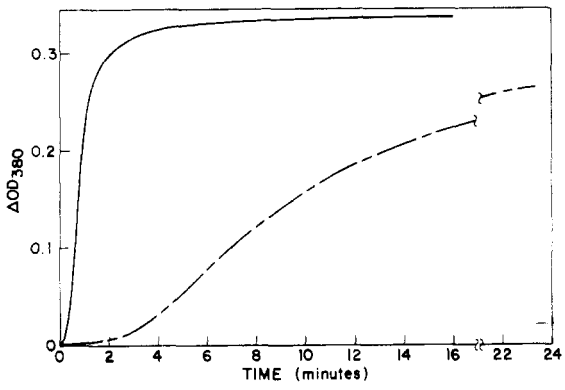

FIGURE 1: Microtubule assembly with AMP-PNP. GDP/free tubulin (2.4 mg/mL) containing substoichiometric amounts of GDP at the E site was polymerized in purification buffer at 37 °C. The polymerization reaction was monitored turbidimetrically: (— - —) 2 mM AMP-PNP; (—) 0.2 mM GTP.

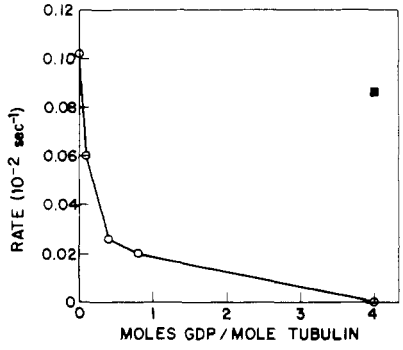

FIGURE 2: Inhibition by GDP and ADP of microtubule assembly with AMP-PNP. GDP/free tubulin (2.9 mg/mL) containing substoichiometric amounts of GDP at the E site was incubated for 2 min at 37 °C with increasing concentrations of GDP or with a single concentration of ADP. Polymerization was induced at 37 °C by addition of 1.5 mM AMP-PNP, and the polymerization reaction was monitored turbidimetrically. Initial rate of assembly was quantitated as the largest 100-s increase in the polymerization curve. (O—O) GDP; (■) ADP.

was only slightly inhibitory. The inhibition of AMP-PNP-induced microtubule assembly by GDP but not by ADP suggested that AMP-PNP may promote polymerization by binding to the E site of tubulin.

Nucleotide Concentration Dependence of Microtubule Assembly before and after Removal of GDP. The initial rate of microtubule assembly was used to assay the dependence of the polymerization reaction on nucleotide concentration. Under conditions where phosphorylation of GDP at the E site can be ruled out as a pathway of nucleotide action in microtubule assembly, the initial rate of the polymerization reaction may be interpreted as an indirect measure of the effectiveness of nucleotide interaction with the E site. The concentration dependence of the initial rate of the polymerization reaction may reflect the strength of the binding of the nucleotide to tubulin as well as its potency to induce assembly. Nucleotides were characterized by two rate titration curves, one each for the dependence of the initial rate of polymerization on nucleotide concentration in GDP-tubulin and the other in GDP/free tubulin (Figures 3 and 4). The midpoint of each rate titration curve, which represents the nucleotide concentration at which the initial rate of polymerization was half-maximal, is referred to as the $K_{effective}$ (K_{eff}). The K_{eff}s for all nucleotides examined in this study are listed in Table I.

Comparison of Figure 3a, which represents rate titration curves for rGTP, dGTP, and rGMP-PCP in GDP-tubulin,

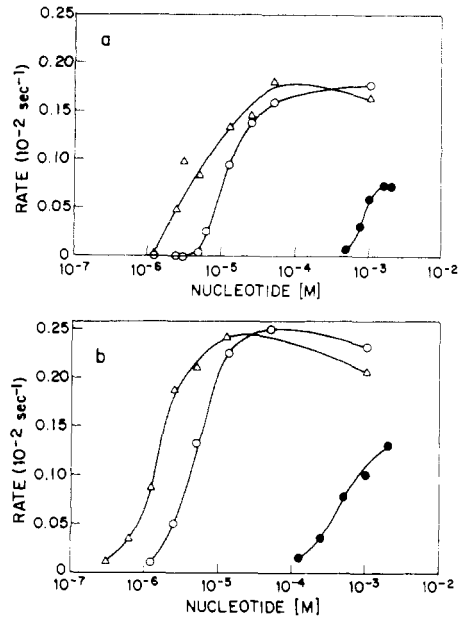

FIGURE 3: Effect of partial E site GDP removal on the dependence of the initial rate of microtubule assembly on GTP, dGTP, and GMP-PCP concentration. Microtubule polymerization at 37 °C was monitored turbidimetrically at various nucleotide concentrations, and the initial rate of assembly was quantitated as the largest 100-s increase in the polymerization curve. Measurements were performed in several separate experiments. (a) GDP-tubulin (3.0 mg/mL) containing stoichiometric amounts of GDP at the E site. (b) GDP/free tubulin (3.2 mg/mL) containing substoichiometric amounts of GDP at the E site. (O—O) GTP; (Δ—Δ) dGTP; (●—●) GMP-PCP.

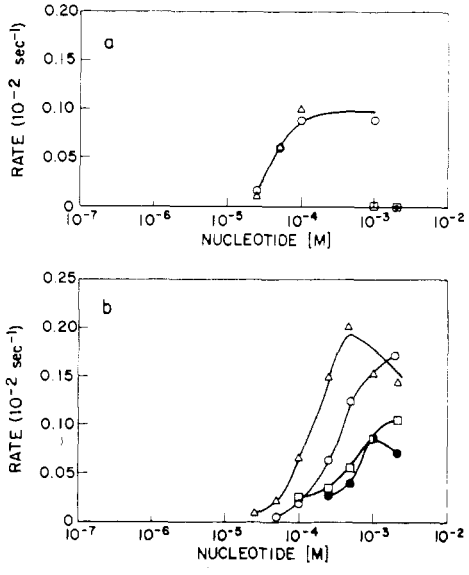

FIGURE 4: Effect of partial E site GDP removal on the dependence of the initial rate of microtubule assembly on ATP, dATP, AMP-PNP, and AMP-PCP concentration. Experimental details were the same as in Figure 3. (a) GDP-tubulin (3.0 mg/mL) containing stoichiometric amounts of GDP at the E site. (b) GDP/free tubulin containing substoichiometric amounts of GDP at the E site (3.2 mg/mL in ATP, dATP, and AMP-PNP curves; 2.2 mg/mL in AMP-PCP curve). (O—O) ATP; (Δ—Δ) dATP; (□—□) AMP-PNP; (●—●) AMP-PCP.

with Figure 3b, which shows rate titration curves for the same nucleotides in GDP/free tubulin, demonstrates that partial removal of GDP from the E site results in a shift of the K_{eff} to two- to threefold lower concentrations of nucleotide, as expected qualitatively from a decrease in competition from GDP for binding to the E site (see also Table I). Figure 3 shows

TABLE I: Values of K_{eff}[a] before and after Partial Removal of GDP from the E Site.[b]

	K_{eff} (μM)	
	GDP-tubulin[c]	GDP/free tubulin[d]
rGTP	12	5
dGTP	5	2
rGMP-PCP	810	480
rATP	48	360
dATP	48	180
rAMP-PNP	No assembly	440
rAMP-PCP	No assembly	500
rCTP	60	2000
dCTP	60	680
rUTP	48	490
rITP	49	100

[a] Defined as the nucleotide concentration at which the initial rate of microtubule assembly is half-maximal. [b] Protein concentration was approximately 3.0 mg/mL in all determinations. [c] Contained stoichiometric amounts of GDP at the E site. [d] Contained substoichiometric amounts of GDP at the E site (see text).

TABLE II: Extent of Microtubule Assembly with Substoichiometric Levels of dGTP.[a]

dGTP added (μM)	Microtubules formed (μM)[b]
None	1.5
0.1	1.6
0.3	4.5
1.0	6.6
2.0	6.4
8.0	9.3

[a] dGTP was added to GDP/free tubulin (2.8 mg/mL) containing reduced amounts of GDP at the E site. Following incubation for 65 minutes at 37 °C, the total concentration of microtubules formed was determined by quantitative electron microscopy (Kirschner et al., 1975). [b] Microtubules (1 μM) are taken to be equivalent to 0.11 mg/mL.

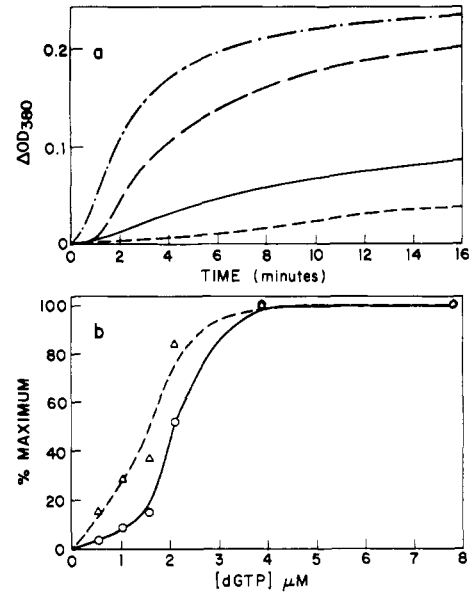

FIGURE 5: Microtubule assembly with substoichiometric concentrations of dGTP as assayed by turbidimetry. GDP/free tubulin (2.5 mg/mL) containing substoichiometric amounts of GDP at the E site was polymerized with increasing concentrations of dGTP at 37 °C. The polymerization reaction was monitored turbidimetrically. (a) Turbidimetric polymerization curves. (- - -) 0.54 μM dGTP; (—) 1.6 μM dGTP; (— — —) 2.1 μM dGTP; (— • —) 3.9 μM dGTP. (b) Dependence of assembly on nucleotide concentration. (O—O) Initial rate, quantitated as the largest 100-s increase in the polymerization curve. (Δ - - - Δ) Total polymerization as judged by plateau level of curve after 16 min.

further that dGTP is two to three times more effective than rGTP, as judged from a comparison of their K_{eff} in GDP/free tubulin and in GDP-tubulin (Table I). Finally, Figure 3 indicates that substoichiometric concentrations of dGTP and rGTP promote half-maximal polymerization rates in GDP/free tubulin at nucleotide:tubulin stoichiometries of 0.06:1.0 for dGTP and 0.17:1.0 for rGTP. K_{eff} values were not determined for GMP-PNP due to variability in polymerization rates obtained with this analogue (Penningroth & Kirschner, 1977; Penningroth, 1977), presumably due to impurities.

Figure 4 shows that the rate titration curves for rATP and dATP gave quite different results. The required nucleotide concentrations in GDP/free tubulin (Figure 4b) are higher as compared with GDP-tubulin (Figure 4a). Further, rATP and dATP gave identical rate titration curves in GDP-tubulin (Figure 4a), but dATP was found to be twofold more effective than rATP in GDP/free tubulin (Figure 4b). These results were obtained by comparing rATP with dATP in the same protein preparations in order to rule out possible differences in initial polymerization rates due to variability in the preparation of either GDP/free tubulin or GDP-tubulin. The shift of the K_{eff} to higher concentrations of rATP and dATP (Table I) and the enhanced effectiveness of dATP compared with rATP (Figure 4 and Table I) suggested that the partial removal of GDP from the E site may have resulted in a change in the mechanism of nucleotide action in microtubule assembly from phosphorylation of GDP bound at the E site to direct interaction with the E site. Figure 4 shows, finally, that in GDP/free tubulin, nonhydrolyzable ATP analogues were capable of inducing microtubule assembly about half as rapid as that induced with rATP. No polymerization was observed with AMP-PNP or AMP-PCP in GDP-tubulin, while dATP and rATP were equally effective.

The K_{eff} values for rCTP, dCTP, UTP, and rITP were also shifted to higher nucleotide concentrations by the partial removal of GDP (Table I). Similarly, the K_{eff}s for dCTP and rCTP were identical in GDP-tubulin, while the K_{eff} for dCTP was three fold lower than the K_{eff} for rCTP in GDP/free tubulin (Table I). These results, like those for rATP and dATP in Figure 4, suggest that partial GDP removal resulted in a change in the mechanism of action of weakly binding nucleotides from GDP phosphorylation to direct binding to the E site. Comparison of the effectiveness of dGTP, dATP, and dCTP with that of rGTP, rATP, and rCTP, respectively, suggests that deoxyribonucleotides bind more strongly to tubulin or are more potent in the polymerization reaction than are ribonucleotides (Table I).

Microtubule Assembly at Substoichiometric Levels of Nucleoside Triphosphate. Turbidimetric polymerization curves obtained in GDP/free tubulin with substoichiometric concentrations of dGTP ranging from 0.02 to 0.35 mol/mol are shown in Figure 5a. When the initial rate of polymerization is plotted as a function of dGTP concentration (Figure 5b), a sigmoidal curve is obtained with a half-maximal concentration of dGTP of 2.1 μM or 0.09 mol/mol.

The turbidimetric assay for initial polymerization rate is not sensitive to very low levels of microtubule assembly. Therefore, microtubule assembly with substoichiometric concentrations of dGTP was assayed by quantitative electron microscopy (Kirschner et al., 1975), where the amount of tubulin in morphologically identifiable microtubules can be determined. The results in Table II show that at a concentration of tubulin of

2.8 mg/mL (25 μM), 0.1 mg/mL tubulin or 7% of the total tubulin was assembled into microtubules on warming to 37 °C in the absence of any added nucleoside triphosphate. However, no microtubules whatsoever were observed in the absence of added nucleoside triphosphate if GDP was not removed from the E site of tubulin. Table II also shows that, at 3×10^{-7} M dGTP, microtubule assembly was stimulated to a level of 0.5 mg/mL (19% of the total tubulin). A concentration of 3×10^{-7} M dGTP represents a molar ratio of 0.012 mol of dGTP per mol of total tubulin. The stoichiometry can be expressed in terms of the proportion of tubulin capable of polymerization at saturating dGTP levels, which is the most conservative estimate for active subunits. Table II shows that not more than 40% of the tubulin subunits were assembled into microtubules in that experiment. A concentration of 3×10^{-7} M added dGTP therefore stimulated polymerization of 50% of the polymerizeable tubulin, and the nucleotide:tubulin stoichiometry at 50% polymerization was 0.03 mol of dGTP per mol of polymerizable tubulin. Plateau levels of microtubule assembly seem to have been approached at 1 μM dGTP (0.04 mol of dGTP per mol of total tubulin or 0.1 mol of dGTP per mol of polymerizeable tubulin). Thus, at 1 μM dGTP, the concentration of tubulin in microtubules was 7 μM (Table II). In a separate experiment, GDP/free tubulin was also found to polymerize with a pyruvate kinase–phosphoenolpyruvate GTP generating system; controls showed no polymerization with either pyruvate kinase or phosphoenolpyruvate alone. This result provided further evidence that microtubule assembly may be induced by the binding of substoichiometric levels of GTP to the E site, since only an estimated 40% of the tubulin retained exchangeably bound GDP in GDP/free tubulin.

Discussion

Removal of GDP from the E Site. A method for removing GDP from the E site of microtubule protein is described in this report. This method consists of two steps: (1) the displacement of GDP from the E site by competitive binding with 50-fold molar excess of GMP-PCP; and (2) the subsequent removal of GMP-PCP on a gel filtration column. Exposing the protein to 0.75 M NaCl prior to gel filtration aided the removal of GMP-PCP. Preparations obtained by this procedure typically had a nucleotide:tubulin ratio of 1.0:1.0, and they polymerized in response to AMP-PCP. When the salt step was omitted in a previous procedure (Penningroth et al., 1976), the protein had a variable nucleotide:tubulin ratio of 1.0:1.0 to 1.3:1.0, and microtubule assembly could not be reproducibly effected with AMP-PCP in these preparations. The method described here for GDP removal did not cause tubulin denaturation, as judged by the ability of microtubule protein to polymerize efficiently in response to GTP (Figure 1).

The difficulty in removing GDP from the E site is not surprising in view of the ligand retention effect for gel filtration and dialysis as described by Silhavy et al. (1975). Given a K_{diss} for the GDP-tubulin complex of 1.5×10^{-7} mol L^{-1} (Jacobs & Caplow, 1976) and concentrations of purified tubulin on the order of 2–5 mg/mL, it can be calculated that only a small percentage of the E site GDP is removed by simple gel filtration (Dixon, 1976). Another factor which may contribute to the stabilization of the GDP-tubulin complex in vitro is the presence of trace amounts of glycerol, which was found by Arai et al. (1975) to depress the dissociation rate of GTP from tubulin.

Although the method described here for removing GDP resulted in a nucleotide:tubulin stoichiometry of 1.0:1.0, GDP removal was not complete, as judged by the capacity of the phosphoenolpyruvate–pyruvate kinase GTP generating system to promote microtubule assembly in the absence of added GDP, as well as by evidence from thin-layer chromatography that tubulin-associated nucleotides contained some GDP. The nucleotide:tubulin stoichiometry does not seem to provide an accurate estimate of the amount of nucleotide bound to the E site, possibly as a result of the presence of an undetermined amount of inactive protein which has the effect of lowering the measured nucleotide:tubulin ratio. This possibility is supported by previous reports that tubulin prepared by other methods may bind only 0.7–0.9 mol of nucleotide at the E site (Arai et al., 1975; Kobayashi, 1975). We interpret our results as suggesting that our preparations of GDP–tubulin having a total nucleotide:tubulin ratio of 1.4:1.0 may contain 0.7 mol of GTP at the N site and 0.7 mol of GDP at the E site, and that our preparations of GDP/free tubulin having a nucleotide:tubulin ratio of 1.0:1.0 may contain 0.7 mol of GTP at the N site and 0.3 mol of GDP at the E site. This interpretation, if correct, suggests that we were able to remove approximately 60% of the GDP from the E site. This interpretation is supported by estimates of tubulin-associated nucleotides by thin-layer chromatography, which indicate that the ratio of GDP to GTP is reduced by 60–80% in GDP/free tubulin as compared with GDP–tubulin. Previous work has shown that GTP associated with tubulin can be treated as a marker for estimating removal of nucleotide from the E site, since GTP from the N site neither exchanges nor turns over in vitro, while any GTP bound at the E site is rapidly hydrolyzed to GDP (Kobayashi, 1975; Penningroth et al., 1976).

Nucleotide Specificity of the E Site. The results are consistent with a previous suggestion (Penningroth et al., 1976; Penningroth & Kirschner, 1976, 1977) that GTP, GMP-PCP, and GMP-PNP may induce microtubule assembly by merely binding at the E site. Nucleotide binding might induce a conformational change which renders tubulin capable of incorporation into the microtubule lattice. Alternatively the nucleoside triphosphate itself might supply the proper local interactions between subunits. In this paper we have presented evidence that the E site possesses broad specificity for nucleoside triphosphates, and that, in the absence of competition from GDP, any nucleoside triphosphate can induce polymerization by binding to the E site. Two arguments support this conclusion.

(1) AMP-PNP promoted microtubule assembly at about 50% of the rates observed with ATP in GDP/free tubulin (Figure 4b), while no polymerization occurred in GDP tubulin. This suggests that polymerization with hydrolyzable nucleoside triphosphates in GDP/free tubulin can also be accounted for quantitatively by a mechanism of direct interaction with the E site.

(2) In GDP–tubulin, dGTP was found to stimulate microtubule assembly at a two-fold lower concentration than rGTP (Table I), suggesting that the E site has a greater affinity for, or interacts more favorably with, deoxyribonucleotides than ribonucleotides. No difference, however, was observed between dATP and rATP or between dCTP and rCTP in GDP–tubulin, suggesting that the NDP-kinase mechanism of assembly is equally specific for ribo- and deoxyribonucleotides. However, following the partial removal of GDP from the E site of tubulin (GDP/free tubulin), dATP and dCTP were found to stimulate assembly at lower concentrations than rATP and rCTP, respectively (Table I). This result suggests that in GDP/free tubulin, dATP, rATP, dCTP, and rCTP, like dGTP and rGTP, promote assembly by direct interaction with the E site.

The inhibitory effect of GDP on microtubule assembly has been noted previously (Weisenberg et al., 1976; Penningroth

& Kirschner, 1977). It is demonstrated here that polymerization with nonhydrolyzable ATP analogues is prevented either when GDP is not removed from the E site (Figure 4a) or when GDP is added back to GDP/free tubulin (Figure 2). These observations indicate that under most in vitro conditions, nucleoside triphosphates other than GTP and its analogues cannot interact directly with tubulin because the E site contains tightly bound GDP. High concentrations of weak ligands such as ATP may compete with GDP for binding to the E site (Arai et al., 1975). However, because all nucleoside triphosphates begin to inhibit microtubule assembly above concentrations of about 5 mM, apparently as a result of a nonspecific dissociation of 36 S rings (Penningroth & Kirschner, 1977), microtubule assembly in vitro cannot be induced with high concentrations of weak nucleotide ligands in the presence of GDP at the E site. Rather, the presence of GDP at the E site appears to have the effect of forcing weakly binding nucleoside triphosphates to promote polymerization by an indirect mechanism via the NDP-kinase. This interpretation is supported by the results in Figures 3 and 4 and Table I. Whereas removal of bound GDP makes the interaction with GTP more favorable, shifting the K_{eff} from 12 μM to 5 μM, it causes a marked decrease in the effectiveness of ATP, shifting K_{eff} from 48 μM to 360 μM. This shift is consistent with the explanation that the preferred pathway of ATP action is by the NDP-kinase reaction, and removal of GDP from tubulin inhibits that pathway. However, since GDP is removed partially from the E site in GDP/free tubulin, all nucleoside triphosphates can interact directly with the E site of tubulin at concentrations low enough to permit microtubule polymerization in vitro.

Mode of Nucleotide Action in Microtubule Assembly. The findings presented in this report are consistent with an allosteric view of nucleotide action in microtubule assembly. In this view, which has been proposed before by Kobayashi & Simizu (1976) and by us (Penningroth et al., 1976; Penningroth & Kirschner, 1977), nucleotides are envisioned as allosteric regulators which act to shift the polymerization equilibrium by stabilizing tubulin in a conformation which is either favorable or unfavorable to assembly. This concept has been treated theoretically by Oosawa & Asakura (1975). An allosteric mechanism can explain the effectiveness of various nucleoside triphosphates at promoting microtubule assembly under various conditions. Several papers have suggested that guanosine nucleoside triphosphates (GTP, dGTP, GMP-PCP, and GMP-PNP) are able to promote polymerization by direct binding to tubulin (Arai & Kaziro, 1976; Weisenberg et al., 1976; Penningroth et al., 1976; Figure 3). Presumably, these nucleotides have relatively large association constants and can compete with GDP for binding to the E site. By contrast, adenosine, cytosine, uridine, and inosine triphosphates appear to have a relatively low affinity for tubulin (Figure 4 and Table I), and hence, in the presence of GDP, they cannot act to shift the equilibrium sufficiently to an active state to induce assembly by binding to the E site. Nucleoside triphosphates at concentrations above 5 mM inhibit assembly, and therefore higher concentration ranges cannot be studied. Evidence presented elsewhere indicates that noninhibitory concentrations of weakly binding nucleoside triphosphates can act indirectly by a separate mechanism via an NDP-kinase (Kobapashi & Simizu, 1976; Weisenberg et al., 1976; Penningroth & Kirschner, 1977). The results reported here suggest that the effect of the NDP-kinase may be interpreted as one of lowering the concentration of bound GDP and raising the concentration of bound GTP. Thus, the NDP-kinase affects the ratio of tubulin-bound GTP to GDP at the E site, and, in the view presented here, it is this ratio which determines the rate of assembly. If GDP is partially removed from the E site, thus lowering the GDP to substoichiometric levels, the proportion of active subunits can be increased by the direct binding of weak ligands to the E site. It is possible that allosteric constants or binding constants may be sensitive to solvent effects. This would explain the transition from inactive to active tubulin effected by glycerol or sucrose even in the presence of GDP at the E site (Shelanski et al., 1973).

Evidence that an unliganded active tubulin state actually exists was provided by the observation that, following partial removal of GDP, microtubules can polymerize without the addition of nucleoside triphosphates (Table II), whereas no spontaneous polymerization occurred if tubulin contained stoichiometric amounts of GDP. This observation suggests that tubulin may alternate between two conformational states, an active state (promoted by binding nucleoside triphosphates) and an inactive state (promoted by binding GDP).

A purely allosteric model for assembly, while successful for GMP-PCP-induced polymerization, does not take into account the role of GTP hydrolysis at the E site which occurs during polymerization. Weisenberg et al. (1976) and Weisenberg & Deery (1976) have suggested that GTP hydrolysis may be required for microtubule disassembly and that therefore the energy of GTP hydrolysis may be used to promote destabilization of the microtubule. While this is consistent with the view presented here, we feel that the magnitude of this effect has been overestimated (Penningroth & Kirschner, 1977), and that the destabilizing effect of hydrolysis on the microtubule is much smaller than would be expected if the free energy of GTP hydrolysis were coupled to microtubule destabilization. Experiments designed to test whether or not an allosteric model is appropriate for microtubule disassembly and to assess the role of GTP hydrolysis in this process could be complicated by slow rates of allosteric interconversion as suggested by experiments showing hysteresis in the assembly reaction (Gaskin et al., 1974). In this discussion, therefore, we have only considered the effect of nucleotides on a pre-equilibrium before assembly. Whether a totally allosteric view is appropriate for the entire assembly-disassembly reaction, including E-site GTP hydrolysis and turnover within the microtubule, is unclear at present.

Significance of the Substoichiometric Nucleotide Action in Microtubule Assembly. As shown in Table II and Figure 5, maximum levels and rates of polymerization are attained at substoichiometric concentrations of dGTP. Microtubule polymerization with substoichiometric amounts of GTP has also been reported by MacNeal et al. (1977). Table II shows that, at 1 μM dGTP, the concentration of microtubules polymerized is 6.6 μM. Similarly, the initial rate of assembly was found in Figure 5 to reach the maximum level at 0.16 mol of dGTP added per mol of tubulin. In these experiments, only about 40% of the tubulin polymerized at high dGTP concentrations (9.3 μM out of 25 μM polymerized at 8 μM dGTP, see Table II). Even expressed as total polymerizable tubulin, the maximum rate of assembly is attained at 0.4 mol of dGTP per mol of polymerizable tubulin. These results suggest that not all tubulin subunits need bind GTP in order to be incorporated into the microtubule lattice. In Table II at 1 μM dGTP, only 1 out of 7 of the tubulin molecules in the microtubule has bound GTP during polymerization. Table II shows clearly that substoichiometric amounts of dGTP do *not* act catalytically and are *not* regenerated in a phosphorylation/dephosphorylation cycle during microtubule assembly, as proposed by MacNeal et al. (1977). In their experiments, substoichiometric amounts of GDP were reported to be present but there was a large reservoir of high energy phosphate available which could

continuously regenerate GTP. In our experiment, substoichiometric amounts of dGTP were added with no capacity to generate more high energy phosphate components. The presence of any nucleoside triphosphate hydrolysis activity merely accentuates the substoichiometric effect. Thus, substoichiometric concentrations of nucleotide–tubulin complex can induce polymerization. Whether a polymer nucleus forms which consists entirely of activated subunits or of a mixture of activated and unactivated subunits, and what polymeric form the activated tubulin molecules are in, cannot be determined from these data.

Evidence that nucleotide binding may be involved in a cooperative step in the initial stage of microtubule assembly was provided by the observation that a plot of initial rate vs. the total dGTP concentration in Figure 5b gave a sigmoidal curve. Due to the high affinity of GTP for tubulin, at substoichiometric levels all of it may be considered bound. It should be possible to obtain some notion of the number of activated tubulin subunits in a polymer nucleus from the slope of a plot of the log of the initial rate of assembly vs. the log of the nucleotide concentration, assuming that the initial rate of polymerization depended solely on the concentration of tubulin subunits containing bound nucleoside triphosphate. If the nucleus consisted exclusively of activated subunits, then the slope of the log–log plot for stoichiometrically binding ligands would give the order of the nucleation step. The slopes of log–log plots of data in Figure 3b and Table II were found to be 1.7 and 1.5 for GTP and dGTP. The dependence of initial rate on roughly the square of the GTP or dGTP concentration suggests that a higher order reaction involving at least two tubulin subunits containing bound nucleoside triphosphate may be involved in some nucleation step in the assembly reaction.

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