Assembly of Purified GDP–Tubulin into Microtubules Induced by Taxol and Taxotere: Reversibility, Ligand Stoichiometry, and Competition†

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ABSTRACT: Purified tubulin fully liganded to GDP at the exchangeable nucleotide binding site has been prepared by a new direct nucleotide exchange procedure. This normally inactive GDP–tubulin is driven to assemble into microtubules by the binding of the antitumor drug taxol or its more soluble side-chain analogue Taxotere in Mg^{2+}-containing buffer, and it disassembles by cooling the solution. Therefore this ligand-induced equilibrium microtubule assembly system dispenses with the requirement of a γ-phosphate–metal cation ligand bound at the nucleotide site for tubulin to be active. GDP–tubulin can also form characteristic pseudoordered aggregates of double rings. These aggregates dissociate upon warming or by addition of GTP. Back-substitution of the nucleotide γ-phosphate permits glycerol-induced assembly without taxol and reduces the critical protein concentration required for drug-induced microtubule assembly by a factor of 2.6 ± 0.1. The ligand-induced assembly is maximal at taxol or Taxotere concentrations equimolar with tubulin, and both drugs bind to assembled tubulin with a stoichiometry of 0.99 ± 0.04 ligand per $\alpha\beta$ dimer. Taxotere apparently competes with taxol for the same binding site, with 1.9 ± 0.1 times larger effective affinity. Similarly, the Taxotere-induced assembly of GDP–tubulin or GTP–tubulin proceeds with a critical protein concentration 2.1 ± 0.1 times smaller than with taxol.

Microtubules are noncovalent helical polymers of tubulin, a $2 \times 50\,000$ M_r $\alpha\beta$ heterodimer. Microtubule-associated proteins and microtubule motors bind to the microtubule wall, making possible the regulation of these dynamic structures and their interaction with other eukaryotic cell components. In dividing cells, microtubules are essential for chromosome segregation (Mitchinson & Sawin, 1990), and they constitute the target of antimitotic drugs (Hamel, 1992). Tubulin contains an exchangeable GTP/GDP binding site at the β subunit (E site), which becomes nonexchangeable in the assembled state. GTP–tubulin is the active form of the protein, and GDP–tubulin is inactive in microtubule assembly. Since GTP is hydrolyzed and phosphate released as a consequence of microtubule assembly, the body of the microtubule consists of GDP–tubulin, which is intrinsically unstable, and is prevented from fast diassembly by interaction with the GDP–tubulin–P_i and GTP–tubulin terminal cap (Carlier, 1991). The stochastic loss and recovery of the stabilizing cap generate the length fluctuations characteristic of microtubule dynamic instability (Bayley et al., 1990).

The antitumor drug taxol is a complex diterpen isolated in limited amounts from the bark of the Pacific yew (genus *Taxus*) (Wani et al., 1971; Rowinsky et al., 1990). Taxol has the unique property of inducing the assembly of tubulin into microtubules (Schiff et al., 1979), thus blocking normal microtubule dynamics and cell division (Schiff & Horwitz, 1980). Taxol is experimentally employed to isolate microtubule proteins from scarce sources (Vallee & Collins, 1986). Taxol binds preferentially to microtubules more than to unassembled tubulin and induces assembly by means of an unknown mechanism (Parness & Horwitz, 1981; Carlier & Pantaloni, 1983; Howard & Timasheff, 1988; Takoudju et al., 1988a,b). The solution structure of taxol-induced microtubules has been recently analyzed to 3-nm resolution (Andreu et al., 1992). It was reported that taxol-induced assembly of tubulin proceeds in the absence of exogenous GTP (Schiff & Horwitz, 1981; Howard & Timasheff, 1988), in GDP buffers (Carlier & Pantaloni, 1983; Andreu et al., 1992), and also with GDP-containing tubulin (Williams & Rone, 1989). This suggests that the binding of taxol may drive the inactive GDP–tubulin into assembly. However, these types of preparations should still contain substantial GTP–tubulin, as confirmed by the present study. Therefore it was not proven whether the ligand would completely induce assembly of pure GDP–tubulin or only the growth of polymerization nuclei formed from GTP–tubulin.

Taxotere[1] is a new, more soluble semisynthetic drug related to taxol. It is prepared from 10-deacetylbaccatin III, which is extracted from the renewable needles of the English yew (Mangatal et al., 1989; Guéritte-Voegelein et al., 1991; Swindell et al., 1991). Taxotere effectively induces microtubule polymerization and stops cell division (Ringel & Horwitz, 1991; Barasoaín et al., 1991), and it is undergoing promising clinical trials (Bissery et al., 1992). The purposes of this study were to know whether assembly of pure GDP–tubulin into microtubules can be induced by these two ligands and whether both ligands bind to the same site on microtubules

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[1] Abbreviations: GTP, guanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; E site, exchangeable nucleotide binding site of tubulin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PEDTA, 10 mM phosphate buffer containing 1 mM EDTA, pH 7.0; SDS, sodium dodecyl sulfate; GAB, 3.4 M glycerol in 10 mM phosphate buffer containing 1 mM EGTA, 6 mM $MgCl_2$, and 1 mM GTP, pH 6.5; taxol, 4,10-diacetoxy-2α-(benzoyloxy)-5β,20-epoxy-1,7β-dihydroxy-9-oxotax-11-en-13α-yl (2*R*,3*S*)-3-[(phenylcarbonyl)amino]-2-hydroxy-3-phenylpropionate; Taxotere (trademark of Rhône-Poulenc, RP56976), 4-acetoxy-2α-(benzoyloxy)-5β,20-epoxy-1,7β,10β-trihydroxy-9-oxotax-11-en-13α-yl (2*R*,3*S*)-3-[(*tert*-butoxycarbonyl)amino]-2-hydroxy-3-phenylpropionate; HPLC, high-performance liquid chromatography.

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and to determine the exact stoichiometry and relative affinity of their interactions.

MATERIALS AND METHODS

Protein and Chemicals. Tubulin was prepared from calf brain by the modified Weisenberg procedure (Weisenberg et al., 1968; Lee et al., 1973; Andreu et al., 1984), stored in liquid nitrogen, and equilibrated in the desired buffer before use. Tubulin concentrations were determined spectrophotometrically by employing an extinction coefficient of 107 000 M^{-1} cm^{-1} at 275 nm in 10 mM phosphate buffer containing 1% SDS, pH 7 (Andreu et al., 1984), and employing a Hitachi U2000 spectrophotometer. Low tubulin concentrations were measured spectrofluorometrically by excitation at 280 nm (Andreu & Muñoz, 1986), employing a Shimadzu RF-540 fluorometer (excitation slit 5 nm) calibrated with standards of known concentration prepared from the same tubulin.

GTP (dilithium salt) was from Boehringer, and GDP (disodium salt) was obtained from Pharmacia (lots A0000-00-92 and AD1900104), Boehringer, and Sigma (type I). [8-^{3}H]GDP was from Amersham (lot TRK335, batch 38) and was dried before use. Dimethyl sulfoxide and ethanol (both spectroscopic grade), glycerol, sodium phosphate, EDTA, $MgCl_2$, and uranyl acetate were from Merck. EGTA was from Sigma, and tetrabutylammonium dihydrogen phosphate was from Aldrich.

Taxol was kindly provided by the National Cancer Institute, NIH (Bethesda, MD). It was dissolved in dimethyl sulfoxide and kept at −20 °C, and its concentration was measured spectrophotometrically by employing the extinction coefficient $\epsilon_{273} = 1700$ M^{-1} cm^{-1} (Wani et al., 1971; a new determination gave essentially the same value). [^{3}H]Taxol (specific activity 2.29×10^{14} Bq mol^{-1}; a 1.61×10^{-4} M solution in methanol) was a gift from Drs. I. Ringel, The Hebrew University (Jerusalem), and S. B. Horwitz, Albert Einstein College of Medicine (Bronx, NY); an aliquot was dried, dissolved in a working solution of unlabeled taxol in dimethyl sulfoxide to a final concentration of 9.66 mM and a final specific activity of 3.82×10^{12} Bq mol^{-1}, and stored at −20 °C. Taxotere (RP 56976) and [^{14}C]Taxotere (RP 71626) were kindly provided by Dr. J.-L. Fabre, Rhône-Poulenc Rorer (92165 Antony, France). Taxotere was dissolved in dimethyl sulfoxide and kept at −20 °C. It was found to be more than 99% homogeneous by HPLC (Supelcosil LC-18-DB, 70% methanol–30% water); its infrared spectrum (in a NaBr pellet in a Perkin-Elmer 1420 spectrometer) was in accordance with the literature (*N*-debenzoyl-*N*-(*tert*-butoxycarbonyl)-10-deacetyltaxol; Mangatal et al., 1989). Aliquots of this compound were dried to constant weight over P_2O_5 (ca. 10% weight loss) and dissolved in ethanol, and ultraviolet absorption spectra were recorded with a Cary 16 spectrophotometer equipped with an automatic slit mechanism. The following extinction coefficients were determined: $\epsilon_{282} = 818 \pm 8$ M^{-1} cm^{-1}, $\epsilon_{273} = 936 \pm 12$ M^{-1} cm^{-1}, and $\epsilon_{228} = 14500 \pm 300$ M^{-1} cm^{-1}. The values for ϵ_{273} and ϵ_{282} are significantly different from the literature values; however, the value at 228–230 nm is coincident with the literature value within experimental error (Magantal et al., 1989). Taxotere concentration was measured spectrophotometrically in ethanol dilutions, employing the determined extinction coefficients and correcting for the contribution of residual dimethyl sulfoxide. An aliquot of [^{14}C]taxotere (labeled at C-3′ with a specific activity of 1.85×10^{12} Bq mol^{-1}; initial radiochemical purity 99.7%) was dissolved in dimethyl sulfoxide to a final concentration of 8.90 mM, and the working solution was stored at −20 °C. The radiochemical homogeneity of labeled taxol and Taxotere, examined by HPLC (Supelcosil LC-18-DB, 70% methanol–30% water) and scintillation counting of the fractions, was better than 95% and 99%, respectively.

Nucleotide and Mg^{2+} Quantification. Nucleotides were extracted from protein samples with $HClO_4$, processed, and quantified by tetrabutylammonium ion-pair HPLC (Supelcosil LC-18-DB) as described by Seckler et al. (1990), employing guanosine at a known concentration as an internal standard. Total guanine nucleotide concentrations in $HClO_4$ were measured spectrophotometrically, employing $\epsilon_{256} = 12\,400$ M^{-1} cm^{-1} (Correia et al., 1987). The GDP bound to the exchangeable site of tubulin was also measured by scintillation counting of protein solutions and buffers, employing [^{3}H]-GDP and appropriate internal standards. Total Mg^{2+} was measured by atomic absorption spectrometry at 285.2 nm with a Perkin-Elmer Model 2380 spectrometer calibrated with $MgCl_2$ standards. The free concentrations of Mg^{2+} were calculated by correcting for the cation binding to phosphate, EDTA, and nucleotide (Tabor & Hastings, 1943; Greenwald et al., 1940; Courtney et al., 1953; Correia et al., 1987); the multiple equilibrium system was solved with a personal computer program (J. F. Díaz, unpublished). The cation amount chelated by tubulin should be below 10^{-4} M under our conditions, according to the binding data of Frigon and Timasheff (1975), and was neglected in the calculations.

Preparation of GTP–Tubulin and Partially Substituted GDP–Tubulin. Standard equilibration of tubulin in 10 mM phosphate buffer containing 0.1 mM GTP, pH 7.0, by means of Sephadex G-25 chromatography (20 × 0.9 cm columns) gave GTP–tubulin preparations which contained 1.88 ± 0.16 guanine nucleotides per tubulin heterodimer, with a GDP/GTP molar ratio of 0.04 (HPLC) and about 70 μM total residual Mg^{2+}. For glycerol-induced assembly, tubulin was chromatographed in Sephadex G-25 columns equilibrated in GAB[1] buffer.

GTP–tubulin and partially exchanged GDP–tubulin were prepared by equilibration in 10 mM phosphate buffer, pH 7.0, immediately followed by addition of either 1 mM GTP or 1 mM GDP (Boehringer), and 6 mM $MgCl_2$ (final pH 6.7) to aliquots of the protein solution (Howard & Timasheff, 1986; Andreu et al., 1992). These GTP–tubulin preparations contained 1.61 ± 0.01 GTP and 0.09 ± 0.01 GDP (HPLC) per tubulin dimer. The GDP–tubulin preparations contained approximately 1.46 GTP and 0.48 GDP (HPLC), and 0.41 ± 0.10 [^{3}H]GDP, per tubulin dimer; that is, they were only half-exchanged at the time of preparation. Alternatively, equilibration of tubulin in 10 mM phosphate buffer containing 1 mM GDP, pH 7.0, by means of chromatography in a drained Sephadex G-25 centrifuge column followed by a gravity column also gave partially exchanged GDP–tubulin preparations with a GDP/GTP ratio of 0.40 (HPLC), containing 0.62 ± 0.09 [^{3}H]GDP per dimer. The exchange-in of GDP increased slowly with time.

Preparation of Fully Substituted GDP–Tubulin. In order to obtain homogeneous preparations of GDP–tubulin, it was necessary to employ a two-step interchange procedure, with GDP from Pharmacia (which contained 2.8% GMP, 96.8% GDP, and 0.4% GTP by HPLC). Sucrose, Mg^{+2}, and GTP were removed by chromatography in a drained centrifuge column of Sephadex G-25 medium (6 × 1 cm) equilibrated in PEDTA[1] buffer–1 mM GDP, pH 7.0, in the cold. GDP, 10 mM, was added to the protein, which was incubated for 10 min on ice. Tubulin was freed of excess nucleotide and reequilibrated in PEDTA–1 mM GDP by a second chroma-

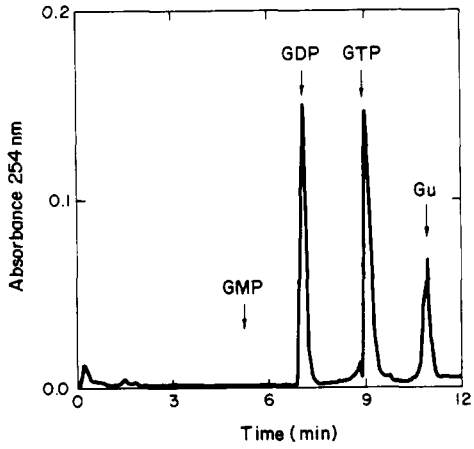

FIGURE 1: HPLC analysis of the nucleotide content of GDP–tubulin, after separation of excess nucleotide by centrifugation in PEDTA buffer containing 7 mM MgCl. Guanosine was employed as an internal standard at a concentration of 15.8 μM. This sample contained 29.1 μM tubulin, 28.6 μM GDP, 28.1 μM GTP, and no detectable GMP.

tography in a cold Sephadex G-25 column (15 × 0.9 cm). At this stage, the total concentration of Mg^{2+} remaining in these preparations was ca. 35 μM. The tubulin samples were routinely centrifuged at 50 000 rpm in TLA 100.2 or TLS 55 rotors for 10 min at 4 °C in a Beckman TL-100 centrifuge prior to addition of 2–8 mM Mg^{2+}, in order to avoid nonspecific sedimentation or turbidity in the assembly assays. Prior to the quantification of the nucleotide contents, aliquots of the final samples were freed of unbound nucleotide, either by chromatography on a Sephadex G-25 column (25 × 0.9 cm) in cold PEDTA–10 μM nucleotide buffer (to avoid loss of exchangeably bound nucleotide) or by two successive centrifugations and washes with nucleotide-free PEDTA–7 mM $MgCl_2$ buffer (2 h at 100 000 rpm and 4 °C in the TLA 100.2 rotor on a Beckman TL-100 centrifuge). Figure 1 shows a characteristic HPLC nucleotide analysis of this nucleotide-exchanged tubulin. These preparations contained 0.96 ± 0.12 GTP (HPLC), 0.95 ± 0.13 GDP (HPLC), 0.99 ± 0.02 [^{3}H]-GDP, and no detectable GMP per tubulin dimer. Their average GDP/GTP ratio was 0.99 ± 0.02 (5 independent determinations); therefore they consist of 99.5 ± 1.0% GDP–tubulin and 0.5% GTP–tubulin at the exchangeable site (assuming equal numbers of exchangeable and nonexchangeable nucleotide sites and that the nonexchangeable nucleotide is GTP). Employing GDP from Boehringer [which contained 6.7% GMP, 90.2% GDP, and 3.1% GTP; a similar composition was obtained for the Sigma GDP, as reported by Zeeberg and Caplow (1979)] resulted in 0.84 ± 0.04 GDP and 1.14 ± 0.06 GTP bound per tubulin dimer and a GDP/GTP ratio of 0.74 ± 0.07, that is, 85 ± 5% GDP–tubulin, in the presence of Mg^{2+}. This indicates that the extent of GDP exchange into the tubulin E site is limited by the purity of the nucleotide employed. Considering that the reported ratio of the relative affinities of GTP–Mg^{2+} and GDP–Mg^{2+} for the E site of tubulin is 3.2:1 (Correia et al., 1987), it can be calculated that 85% is the extent of GDP exchange attainable with these types of commercial GDP preparations. For a GDP preparation with 0.4% GTP (Pharmacia) the calculated GDP exchange is 99.2%. This limitation should apply to any equilibrium nucleotide exchange procedure, once enough Mg^{2+} and GDP are added to the sample [for example, see Williams and Rone (1989)]. The GMP contamination does not interfere, since this nucleotide does not bind appreciably to tubulin (Figure 1). For comparative experiments, GTP–tubulin was obtained simply by addition of 1 mM GTP to GDP–tubulin in Mg^{2+}-containing buffer. This reverse exchange yielded tubulin containing 1.70 ± 0.10 GTP and 0.13 ± 0.05 GDP per dimer, with a GDP/GTP ratio of 0.08 ± 0.04 (i.e., 86 ± 8% GTP–tubulin and 14 ± 7% GDP–tubulin).

Microtubule Assembly. Tubulin samples in the desired buffer were supplemented with $MgCl_2$, and their pH was adjusted if necessary. Prior to assembly, taxol, Taxotere, or dimethyl sulfoxide (typically less than 2.5% volume) was added at 0–2 °C, and the solutions were warmed to the desired temperature. The time needed to reach polymerization equilibrium depends on the tubulin and Mg^{2+} concentrations, the temperature, and the nucleotide bound to protein and varied between 30 min at 37 °C, at high tubulin and Mg^{2+} concentrations, to 3 h at 10 °C. The polymers formed were sedimented at 90000*g* for 10–20 min in a TLA 100 rotor preequilibrated at the temperature of the experiment. The supernatants were separated by aspiration, and the pellets were resuspended in a 1% SDS–10 mM phosphate buffer. Tubulin concentrations in pellets and supernatants were measured as described above. Alternately, tubulin assembly was monitored turbidimetrically at 350 nm. Apparent polymer growth equilibrium constants were estimated as the reciprocal critical concentrations for polymerization, determined at several total protein concentrations (Oosawa & Asakura, 1975; Andreu & Timasheff, 1986). Small aliquots of the assembly solutions were routinely adsorbed to carbon-coated Formvar films on 400-mesh copper grids (for a time in minutes which was roughly equal to the reciprocal of the tubulin concentration in mg/mL), stained for 2 min in 2% uranyl acetate, and observed with Philips 300 and Hitachi 7000 electron microscopes. Microtubule seeds were obtained when required by passing a warm solution of microtubules polymerized with a 1:1 concentration of ligand through a 0.5 × 16 mm needle three times.

Measurement of Ligand Binding to Microtubules. Taxol, Taxotere, or dimethyl sulfoxide was added to tubulin samples in PEDTA buffer containing 1 mM GDP and 4 mM $MgCl_2$, pH 6.7, or in PEDTA buffer containing 1 mM GDP, 1 mM GTP and 8 mM $MgCl_2$, pH 6.7. The samples were incubated for 2 h at 37 °C and centrifuged for 20 min at 50 000 rpm in a prewarmed TLA100.2 rotor (Beckman), employing unused 1-mL polycarbonate tubes. Supernatants were carefully removed by aspiration, and the pellets were resuspended by addition of 1 mL of PEDTA buffer containing 1 mM GDP, pH 7.0, at 0 °C. The tubulin concentration in the pellets and the supernatants was measured spectrofluorometrically, after dilution in SDS. For taxol measurements, an internal standard of taxotere was added to the samples, and vice versa. Samples were dried in a Savant SC-100 Speedvac, and the ligands were extracted by the addition of 100 μL of methanol to the dried pellet. The samples were centrifuged for 15 min in an Eppendorf microcentrifuge, and the supernatants were analyzed in a reverse-phase column (Supelcosil LC-18-DB) in methanol–water (70:30 v/v) at a flow rate of 1 mL/min (Collins & Vallee, 1987). Alternately, ligand binding to microtubules was measured employing [^{3}H]taxol and [^{14}C]-taxotere with tubulin samples incubated and centrifuged as above, but employing 0.2-mL tubes and a TLA100 rotor. Supernatants were removed as above, and the pellets were resuspended by addition of 0.2 mL of 1% SDS–10 mM sodium phosphate buffer, pH 7. [^{3}H]Taxol and [^{14}C]Taxotere concentrations in diluted pellets and supernatants were measured by liquid scintillation counting in a LKB 1219 spectrometer, with the appropriate internal standards. The

stoichiometry of binding was obtained by dividing the ligand concentration by the protein concentration in the diluted pellets. Blanks without protein gave negligible values. The volume of the pellets was very small (always smaller than 2.5% of the total volume) and did not interfere with the measurement, within experimental error.

Ligand Competition. [^{3}H]Taxol and [^{14}C]Taxotere were added to 11 μM tubulin in PEDTA buffer containing 1 mM GDP, 1 mM GTP, and 8 mM $MgCl_2$, pH 6.7, to a total ligand concentration of 20 μM, and the samples were processed as above; the [^{3}H]taxol and [^{14}C]Taxotere concentrations in the pellets and the supernatants were measured by double-channel scintillation counting. Under these conditions the concentration of protein polymerized was constant and was very close (97%) to the total protein concentration. Data were analyzed as a simple competition of two ligands for a single site in assembled tubulin:

$$P + A \overset{K_1}{\rightleftarrows} PA \quad (1)$$

$$P + B \overset{K_2}{\rightleftarrows} PB \quad (2)$$

$$K_1 = [PA]/[P][A] \quad (3)$$

$$K_2 = [PB]/[P][B] \quad (4)$$

$$K_2/K_1 = [PB][A]/[PA][B] \quad (5)$$

where P is the ligand binding site, and A and B are the two ligands. Since the free (supernatant) and bound (pellet) concentrations of taxol and Taxotere were measured, it is possible to determine the value of K_2/K_1 from each data. The complete data set can also be fitted by plotting [PB][A] versus [PA][B] and measuring the slope, which is K_2/K_1 (Peyrot et al., 1992). On the other hand, for experiments performed at constant $[P]_0$ and very close to binding saturation, the value of the concentration of free sites, [P], can be neglected and the following approximation applied:

$$[P]_0 = [PA] + [PB] \quad (6)$$

$$[A]_0 = [A] + [PA] \quad (7)$$

$$[B]_0 = [B] + [PB] \quad (8)$$

$$[A] = [A]_0 - [P]_0 + [PB] \quad (9)$$

$$[B] = [B]_0 - [PB] \quad (10)$$

$$[PA] = [P]_0 - [PB] \quad (11)$$

and substituting eqs 9, 10, and 11 into eq 5,

$$K_2/K_1 = \frac{[PB]([A]_0 - [P]_0 + [PB])}{([P]_0 - [PB])([B]_0 - [PB])} \quad (12)$$

Equation 12 was employed to obtain the value of K_2/K_1 which gave the best least squares fit to the experimental values of [PB] for the data set.

RESULTS

Reversible Assembly of GDP–Tubulin and of GTP–Tubulin into Microtubules Induced by Taxol and Taxotere. A new nucleotide exchange method based on the equilibration of tubulin in excess pure GDP and the simultaneous chelation of Mg^{2+} gave GDP–tubulin preparations in which over 99% of the exchangeable nucleotide is GDP, and the protein contains 1 GDP per $\alpha\beta$ dimer, within experimental error (Figure 1; Materials and Methods). This nucleotide-exchanged tubulin was totally inactive for assembly under standard conditions, but remained equally capable of assembly upon addition of GTP and glycerol as the protein directly equilibrated into GTP and glycerol (not shown), which indicated minimal denaturation. Figure 2 shows the time courses of taxol- and Taxotere-induced assembly of 20 μM GDP–tubulin in phosphate buffer containing 2 mM free Mg^{2+}, started by a temperature jump to 37 °C. GDP–tubulin polymerized nearly as fast and to nearly the same turbidity as GTP–tubulin. Taxotere induced slightly greater and more slowly reversible turbidity than taxol. This assembly was totally reversible by cooling the sample at 0 °C in each case, as monitored by turbidity. However, above approximately 100 μM tubulin or 5 mM free Mg^{2+} there was significant assembly in the cold. Therefore, in order to observe the cold-reversible assembly, low protein and Mg^{2+} concentrations are required. The polymers formed were quantified by sedimentation, and the protein assembly system was shown to conform to a nucleated condensation polymerization (Oosawa & Asakura, 1975). Under the above conditions (4 mM total Mg^{2+}; Figure 2) the tubulin critical concentration values measured were GDP–Taxotere, 4.9 μM; GTP–Taxotere, 3.3 μM; GDP–taxol, 10.6 μM; and GTP–taxol, 7.7 μM. The critical concentration values remained practically unchanged if a 2-fold excess of ligand was employed. The polymers induced by both ligands were microtubules, as shown by the electron micrographs in Figure 3, and few opened microtubular polymers, as also studied by X-ray solution scattering (Andreu et al., 1992, and unpublished results). Addition of 2% GDP–tubulin microtubule seeds to the above experiment reduced by approximately 25% the lag time of polymerization (not shown), confirming the ability of GDP–tubulin–ligand to nucleate assembly.

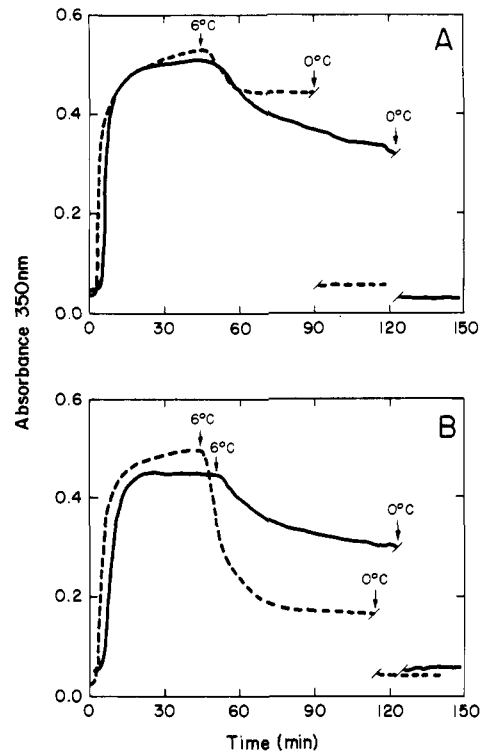

FIGURE 2: Panel A: Time course of assembly of 20 μM tubulin induced by 20 μM Taxotere in PEDTA buffer containing 4 mM $MgCl_2$ and 1 mM GDP, pH 6.7 (continuous line), and in the same solution plus 1 mM GTP (dashed line), monitored turbidimetrically. The assembly was started by warming the samples to 37 °C at time 0. Panel B: Taxol-induced assembly under identical conditions.

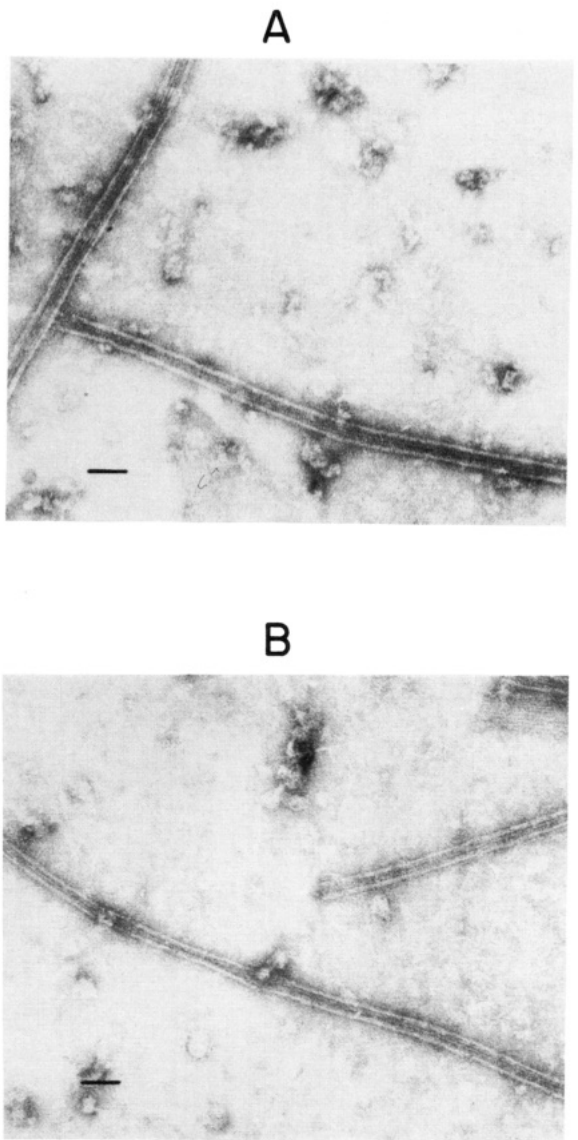

FIGURE 3: Panel A: Characteristic electron micrograph of microtubules induced by Taxotere from GDP–tubulin (PEDTA buffer containing 4 mM $MgCl_2$, and 1 mM GDP, pH 6.7, at 37 °C). Panel B: Taxol-induced microtubules assembled under identical conditions. The bars indicate 50 nm.

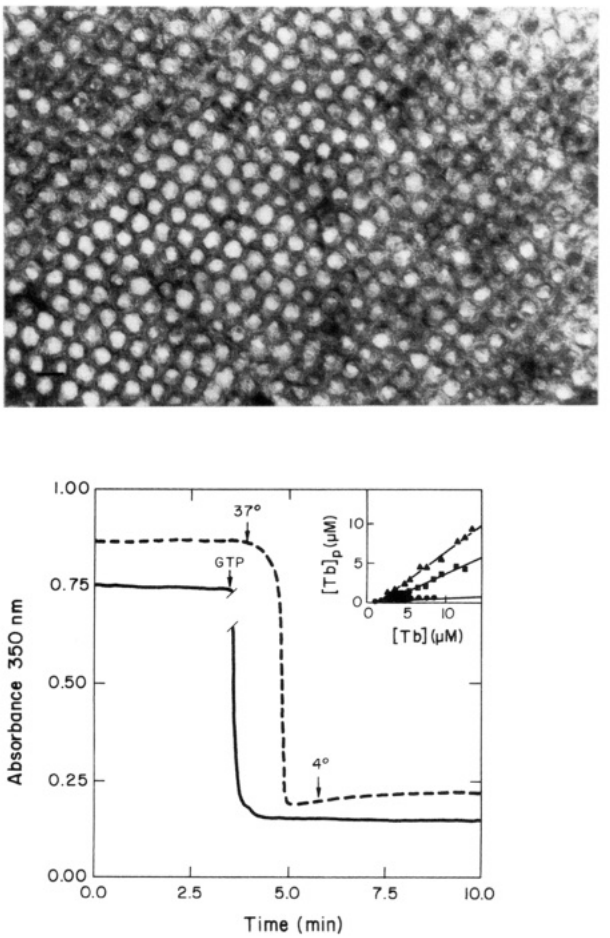

FIGURE 4: Upper panel: Electron micrograph of ring sheets formed by 35 μM GDP–tubulin in PEDTA buffer containing 7 mM $MgCl_2$ and 1 mM GDP, pH 6.7, at 4 °C. The bar indicates 50 nm. Lower panel: Turbidimetrically monitored disassembly of ring sheets of 30 μM GDP–tubulin at 4 °C in the same buffer, induced by the addition of 1 mM GTP (continuous line), and time course of diasssembly of ring sheets formed by 49 μM GDP–tubulin, induced by raising the temperature to 37 °C (dashed line). The inset to the figure is a quantification by sedimentation of the GDP–tubulin polymerization into ring sheets in the same buffer, after different degrees of nucleotide exchange. Circles: 40% GDP–tubulin. Squares: 85% GDP–tubulin. Triangles: 100% GDP–tubulin. *x*-Axis: total tubulin. *y*-Axis: tubulin in pellet.

Mg^{2+}-Induced Formation of Ring Aggregates by GDP–Tubulin. Partial substitution of GDP at the E site of tubulin is known to enhance the Mg^{2+}-induced self-association of tubulin leading to double-ring formation (Howard & Timasheff, 1986). In our case, the homogeneous GDP–tubulin preparations in buffer containing more than 4 mM free Mg^{2+} slowly polymerized at 4 °C to give characteristic double-ring sheets. Above 5 mM free Mg^{2+}, this resulted in a slow ligand-induced polymerization into microtubules upon warming (since the GDP–tubulin rings have to dissociate first) unless assembly was initiated by Mg^{2+} addition to warm tubulin–drug solutions, which prevented the formation of the ring aggregates. As shown by Figure 4, upper panel, the aggregates consisted of a pseudohexagonal close packing of rings, similar to those observed at high Mg^{2+} concentrations (Voter & Erickson, 1979) or in C-terminal subtilisin-cleaved tubulin (Arevalo et al., 1990; Peyrot et al., 1990; Lobert & Correia, 1991), or a pseudoorthogonal close packing not reported before. Actually, both pseudolattices could be observed in the same preparation, as well as apparently mixed type aggregates. The double rings had an outer diameter of ca. 42 nm and an inner diameter of ca. 21 nm. Only the outer rings superimposed in the pseudohexagonal lattice, whereas outer- and inner-ring superposition was observed in the pseudoorthogonal lattice. The GDP–tubulin ring aggregates did not form appreciably below 3 mM free Mg^{2+}, and an apparently amorphous precipitate formed above 16 mM free Mg^{2+}. The aggregates disappeared in a few seconds below 6 mM free Mg^{2+} after the solution was warmed to 37 °C, in a few minutes at 6–10 mM free Mg^{2+} concentrations (Figure 4, lower panel), and in more than 30 min above 10 mM free Mg^{2+}. Upon addition of 1 mM GTP to ring sheet GDP–tubulin suspensions in the cold, the large aggregates disappeared in seconds, as monitored by turbidity (Figure 4, lower panel) and electron microscopy. GTP was fully exchanged into the E site in less than 30 min. The GDP ring aggregates were quantified by centrifugation at different protein concentrations between 1 and 15 μM. Approximately 75% of GDP–tubulin was incorporated into the aggregates, decreasing to approximately 44% and 7% of the protein when partially exchanged 85% and 45% GDP–tubulin were respectively employed (inset to Figure 4, lower panel).

Stoichiometry of Taxol- and Taxotere-Induced Assembly and of the Binding of These Drugs to Microtubules. Figure 5 shows the polymerization of GTP–tubulin at different

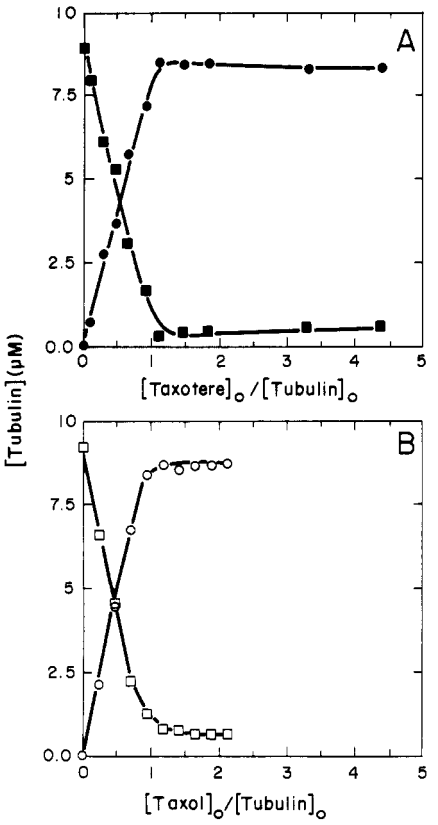

FIGURE 5: Taxol- and Taxotere-induced assembly of tubulin in 10 mM phosphate buffer containing 6 mM $MgCl_2$ and 0.1 mM GTP, pH 6.7, at 37 °C and at different molar ratios of ligand to tubulin, measured by sedimentation. Squares: tubulin concentration in supernatants. Circles: tubulin concentration in pellets resuspended after centrifugation. Panel A: Taxotere. Panel B: taxol. The initial tubulin concentration was 10 μM. The data are corrected for the small concentration of nonspecifically sedimented protein, which was not centrifuged prior to this assay.

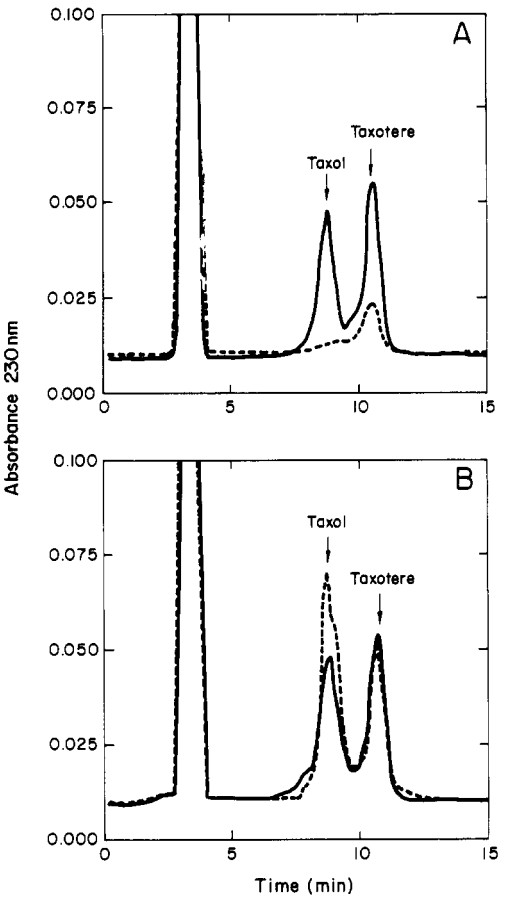

FIGURE 6: Quantification by HPLC of taxol bound to microtubules. Microtubules were induced by taxol at 37 °C from 20 μM tubulin in PEDTA buffer containing 1 mM GDP and 5 mM $MgCl_2$, pH 6.7. Taxotere, 40 μM, was employed as an internal standard, except in the dashed-line sample in the upper panel, in which the Taxotere concentration was 10 μM. Panel A: Total taxol concentration was 18.91 μM, and polymerized tubulin concentration was 18.13 μM. Continuous line: pellet, which contained 17.66 μM taxol. Dashed line: supernatant, which contained 1.25 μM taxol. Panel B: Total taxol concentration was 45.78 mM, and polymerized tubulin concentration was 18.69 μM. Continuous line: pellet, which contained 17.83 μM taxol. Dashed line: supernatant, which contained 27.95 μM taxol.

concentrations of taxol or Taxotere. The drug-induced polymerization increased linearly with the ligand concentration, to reach a maximum at a 1:1 molar ratio of ligand to tubulin $\alpha\beta$ dimer, and remained virtually invariant at greater ligand concentrations. This suggests high-affinity binding of the drugs to one site per assembled tubulin dimer. The taxol-induced microtubule assembly of tubulin has also been shown to be maximal at equimolar ligand concentrations by X-ray solution scattering under the same conditions (Andreu et al., 1992), and reported binding measurements had indicated high-affinity binding of approximately 0.6–0.8 taxol molecule per tubulin dimer in microtubules (Parness & Horwitz, 1981). Therefore we sought to obtain accurate measurements of the taxol and Taxotere binding stoichiometries in our system. Figure 6 shows a quantification by HPLC of taxol bound to microtubule pellets at 0.95:1 (panel A) and 2.3:1 (panel B) total ligand:GDP–tubulin ratios. It can be observed that ligand in excess of 1 molecule per tubulin dimer remained in the supernatant. The stoichiometry in the pellet was essentially 1 taxol or Taxotere bound per tubulin dimer (Table I). At smaller drug concentrations a partial tubulin assembly was obtained, yet any microtubules assembled also contained essentially 1 ligand per tubulin dimer (not shown). Similar experiments were performed in which the binding of radioactively labeled taxol and Taxotere to microtubules were measured, and the results, which are displayed in Table I, also indicated unitary stoichiometry. There were no significant differences between taxol and Taxotere or between GDP–tubulin and GTP–tubulin. The weighted averages of the

Table I: Stoichiometry of Taxol and Taxotere Binding to Microtubules[a]

ligand (method)	GDP–tubulin	GTP–tubulin
taxol (HPLC)	0.95 ± 0.09 (2)	0.99 ± 0.05 (50)
Taxotere (HPLC)	0.95 ± 0.09 (2)	0.95 ± 0.09 (2)
[^{3}H]taxol	1.02 ± 0.02 (4)	1.00 ± 0.03 (20)
[^{14}C]Taxotere	0.99 ± 0.05 (4)	0.99 ± 0.05 (55)

[a] The values given are molecules of ligand bound per assembled tubulin heterodimer, from different determinations (Materials and Methods) at 1–50 μM total tubulin and 1–100 μM total ligand. The ligand:protein molar ratios were approximately between 0.1 and 5. The values are expressed as an average ± the standard deviation (number of determinations in parentheses).

results of the HPLC and radioactive methods, respectively, are 0.99 ± 0.06 and 0.99 ± 0.04 ligand bound per tubulin dimer polymerized.

Competition of Taxol and Taxotere for Binding to Microtubules. Comparison of Ligand-Induced Assembly of GDP–Tubulin and GTP–Tubulin. In order to know whether the site of binding of taxol and Taxotere is the same and the apparent relative affinity of both drugs, both ligands were allowed to interact simultaneously with tubulin. Figure 7

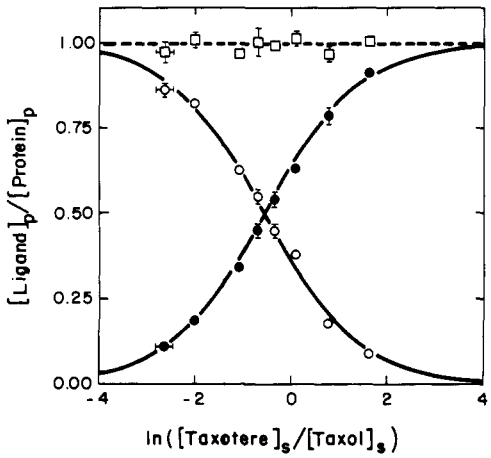

FIGURE 7: Competition between [^{3}H]taxol and [^{14}C]Taxotere for binding to microtubules. Tubulin, 11.3 μM, was assembled at 37 °C in PEDTA buffer containing 1 mM GDP, 1 mM GTP, and 8 mM $MgCl_2$, pH 6.7, by the addition of taxol and Taxotere at a total concentration of 20 μM, at different molar ratios of taxol to Taxotere. The total concentration of microtubules was 11.0 ± 0.10 μM; the tubulin concentration in supernatants (not polymerized tubulin) varied between ca. 0.4 (in taxol excess) and 0.2 μM (in Taxotere excess). Open circles: [^{3}H]taxol bound per polymerized tubulin dimer. Solid circles: [^{14}C]Taxotere bound. Squares: total ligand (taxol plus Taxotere) bound per polymerized tubulin dimer. The solid lines are the best fit to the data, employing a simple competition model of the two ligands for the same site (see Materials and Methods).

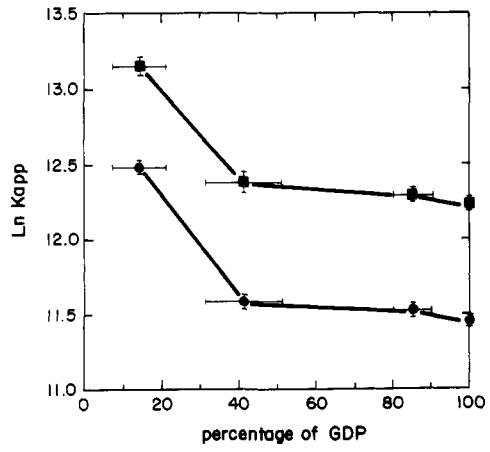

FIGURE 8: Apparent growth equilibrium constants of the ligand-induced polymerization of tubulin at 37 °C in PEDTA buffer containing 4 mM $MgCl_2$ and 1 mM nucleotide, pH 6.7, after different degrees of GDP substitution into the exchangeable site (see Materials and Methods). Squares: Taxotere. Circles: taxol. The ligands were added in a 1:1 molar ratio with tubulin. The data in GTP (1.80 mM free Mg^{2+}) were corrected for the different association constants of GTP and GDP with Mg^{2+} and normalized to the free Mg^{2+} concentration of the GDP data (2.01 mM), employing the Mg^{2+} activity dependence of the critical concentration of this assembly system (Diaz et al., unpublished results). Measurements of the critical concentration at 6 and 8 mM $MgCl_2$ gave the same ratio between taxol- and Taxotere-induced assembly as this experiment.

shows the results of a representative experiment performed with varying concentrations of [^{3}H]taxol and [^{14}C]Taxotere, at a constant total ligand concentration in excess over the tubulin concentration. Under the conditions of this experiment the protein polymerized is close to total and practically constant (i.e., the concentration of binding sites is constant), and the binding sites are essentially saturated. The total ligand (taxol plus Taxotere) bound is 0.99 ± 0.03 ligand per tubulin dimer polymerized. The results fit a simple model in which Taxotere binds to the same site as taxol, with an apparent equilibrium constant 1.86 ± 0.09 times larger than that of taxol (which is indicated by the solid lines in Figure 7).

Figure 8 shows the results of a series of experiments in which the apparent equilibrium growth constants of tubulin assembly, induced by taxol or Taxotere at a 1:1 ratio, were measured as several degrees of exchange of GDP into the tubulin E site. The Taxotere-induced polymerization had a 2.1 ± 0.1 times larger equilibrium constant than the taxol-induced growth reaction, irrespective of the exchangeable nucleotide being GDP or GTP. The presence of the γ phosphate of the nucleotide increased by 2.6 ± 0.1 times the apparent equilibrium growth constant of the assembly induced by both drugs.

DISCUSSION

Ligand-Induced Assembly of GDP-Tubulin into Microtubules. Since it was known that taxol would induce the assembly of tubulin with the exchangeable nucleotide partially substituted by GDP (see introduction), and in order to unequivocally determine the effects of complete nucleotide substitution, a new exchange procedure to prepare GDP–tubulin was devised (see Materials and Methods). This method, which was inspired by the procedure of Seckler et al. (1990) (which gave in our hands about 80% exchange) and by the known Mg^{2+} effects on nucleotide binding described by Correia et al. (1987), allowed over 99% exchange of the tubulin E site. It compares favorably with other reported methods of direct exchange of GDP into the E site of tubulin (Williams & Rone, 1989; Seckler et al., 1990), and its results appear at least equivalent to the preparation of GDP–tubulin by assembly-induced hydrolysis of GTP–tubulin (Mejillano et al., 1990). This essentially homogeneous GDP–tubulin spontaneously underwent Mg^{2+}-induced association to slowly form large double-ring aggregates. These aggregates rapidly disappeared upon warming or GTP addition.[2] However, at high Mg^{2+} concentrations the ring aggregates dissociated very slowly, which limits the feasibility of experiments involving microtubule assembly from GDP–tubulin at high Mg^{2+}.

Tubulin with GDP bound at the E site is inactive under the usual assembly conditions (i.e., in the absence of drug), because (i) it does not spontaneously nucleate assembly and (ii) it does not elongate microtubules, but actually caps the polymer ends (Carlier & Pantaloni, 1982; Carlier et al., 1989). GDP–tubulin has been regarded as being in a "curved" conformation which facilitates ring formation but is different from the active GTP–tubulin "straight" conformation which facilitates microtubule assembly (Howard & Timasheff, 1986; Melki et al., 1989). It has been reasoned that switching from the inactive to the active conformation requires the binding of a properly positioned and coordinated γ phosphate–metal ion ligand at the E site (Shearwin & Timasheff, 1992). The analogues AlF_4^- and BeF_3^- and inorganic phosphate bind to microtubules, stabilizing their structure against depolymerization, and BeF_3^- permits low-rate elongation of preformed microtubules with GDP–tubulin; yet microtubule assembly from the unassembled GDP–protein was not achieved with these ligands (Carlier et al., 1988, 1989). The present report shows unambiguously that taxol and Taxotere efficiently

[2] The latter is in apparent contrast with the report that nucleotide exchange is slow in ring oligomers, because it requires previous ring dissociation (Melki et al., 1989). However, both observations may be the result of the different conditions and may be reconciled by the fact that the ring aggregates appeared at full GDP substitution and were not observed at partial substitution; therefore a relatively small back-exchange of GTP may dissociate the aggregates rapidly.

induce reversible microtubule assembly from the 1:1 GDP–tubulin complex. To our knowledge these are the only tubulin ligands presently reported to fully dispense with the requirement of a γ phosphate at the E site for microtubule assembly. The ligand-induced assembly of purified GDP–tubulin constitutes a simplified microtubule equilibrium system, devoid of the binding, hydrolysis, and release of the nucleotide γ phosphate. Sufficient added linkage free energy must be supplied by the binding of such ligands to shift the overall strength of the polymerization to a level observable at practical protein concentrations (Shearwin & Timasheff, 1992), even from the pure inactive protein form. In order to make GDP–tubulin assemble into microtubules, taxol and Taxotere should interact with tubulin in such a manner that they induce nucleation and elongation from the GDP conformation, stabilizing the GDP–microtubules and making their critical concentration comparable to that of GTP–tubulin.

Stoichiometry of Taxol and Taxotere Binding to Assembled Tubulin. Taxol is believed to stabilize microtubules by binding to the tubulin molecules in the microtubule wall lattice, possibly interacting with dimer–dimer contact zones (see introduction). The results in this report firmly establish that 0.99 ± 0.04 taxol or Taxotere molecule binds per tubulin heterodimer polymerized, apparently at the same site. We have detected no significant binding of taxol or Taxotere to the tubulin heterodimer under nonassembly conditions (unpublished results), which suggests a tight linkage of the ligand binding to the protein polymerization. Both tubulin subunits are homologous and can hardly be distinguished at the resolution of presently reported structural studies, yet they should be distinguished by the binding of taxol in the microtubule. The majority of tubulin–taxol microtubules have a three-start 12-protofilament lattice (Andreu et al., 1992). However, X-ray solution scattering indicates that the microtubules induced by Taxotere again have the typical 13 protofilaments (unpublished results). This structural effect of the polymer should be related to the substitution of the side chain in the ligand analogue, which is a part essential for activity (Guéritte-Voegelein et al., 1991; Swindell et al., 1991). Having one protofilament less implies that either taxol should not bind at the microtubule closure or there should be a mixed lattice of taxol binding sites for this ligand to bind a lateral dimer–dimer contact zones [Figure 10 in Andreu et al. (1992)]. In the case of a lack of binding at the seam, an average stoichiometry of 0.92 taxol bound per tubulin dimer would be expected. However, the data appear to deviate significantly from this value and show no trend when Taxotere is substituted for taxol. The possibility of a mixed lattice at the seam, or a propagated mixed lattice, may be compatible with the unitary taxol binding stoichiometry. A mixed lattice requires the type of lateral contacts between the tubulin monomers to be largely equivalent and requires taxol to bind at homologous and heterologous lateral subunit interfaces. An alternative possibility is that taxol may bind exclusively to one of the tubulin chains (Rao et al., 1992) and that the induction of the dimer–dimer contacts would be an allosteric effect. In this case the ligand binding specificity should be independent of the exact type of microtubule lattice.

Effects of Nucleotide and Ligand Substitution in the Drug-Induced Assembly of Tubulin into Microtubules. Substitution of GTP for GDP into the E site resulted in assembly of microtubules with 2.6 ± 0.1 times smaller critical concentration, which translates into a small difference free energy change of microtubule growth favorable to the GTP-bound protein, $\Delta\Delta G_{app}$(GTP–GDP) = −2.5 ± 0.1 kJ mol^{-1}, regardless of the assembly-inducing drug being taxol or Taxotere. This value has been confirmed under varying solution conditions (Díaz et al., unpublished results).

The drug competition experiments indicated an apparent association constant of Taxotere to microtubules that is 1.9 ± 0.1 larger than that of taxol, which translates to an overall difference binding free energy of binding to microtubules (coupled to polymerization), $\Delta\Delta G_{app}$ (Taxotere–taxol), equal to −1.6 ± 0.1 kJ mol^{-1}. Substitution of Taxotere for taxol favored the overall assembly (coupled to ligand binding) reaction by −1.8 ± 0.1 kJ mol^{-1} favorable to Taxotere, with either GDP or GTP at the E site. This has been confirmed under varying solution conditions (Díaz et al., unpublished results). This difference essentially coincides with the difference obtained in the competition measurements. Therefore Taxotere is a slightly more powerful ligand and assembly inducer than taxol. The thermodynamic properties of this protein assembly system, the linked processes of drug binding and microtubule polymerization, and possible mechanisms of assembly are explored in a forthcoming study.

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