Release of Exchangeably Bound Guanine Nucleotides from Tubulin in a Magnesium-Free Buffer†

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Received November 16, 1983; Revised Manuscript Received July 11, 1984

ABSTRACT: The number of moles of guanine nucleotides (denoted GXP), either guanosine 5′-triphosphate (GTP) or guanosine 5′-diphosphate (GDP), bound to a mole of phosphocellulose-purified tubulin after gel filtration into a variety of nucleotide-free buffers has been measured (H. B. Croom, J. J. Correia, and R. C. Williams, Jr., unpublished results). All buffers we have studied that promote reduction of the number of bound nucleotides to fewer than two per tubulin dimer also eventually cause irreversible loss of activity of the protein. However, in 0.1 M 1,4-piperazinediethanesulfonic acid (pH 6.9) and 2 mM dithioerythritol (with no Mg^{2+}), tubulin rapidly releases approximately 0.4 mol of bound nucleotides during two successive gel filtrations requiring less than 0.5 h and regains the ability to polymerize when magnesium and GTP are immediately added to the buffer. No change in conformation detectable by circular dichroism or sedimentation velocity accompanies this reversible process. (Upon prolonged incubation in the buffer, however, tubulin undergoes irreversible changes according to apparent first-order kinetics with a half-life of approximately 8 h. These changes include the irreversible release of nucleotide, a loss of the ability to polymerize, and a decrease in molar ellipticity between 210 and 240 nm.) The nucleotide which is reversibly released in this buffer comes from that population which exchanges readily with [^{3}H]GTP in vitro. When tubulin purified by phosphocellulose chromatography is incubated in a Mg^{2+}-containing buffer for 30 min with [^{3}H]GTP at concentrations from 70 μM to 1 mM at temperatures from 0 to 24 °C, the difference between the total GXP bound and the [^{3}H]GTP that becomes bound by exchange is consistently 1.2 ± 0.1 per tubulin dimer. These observations indicate that phosphocellulose-purified tubulin is composed of two types of dimers: one (approximately 80% of the total) which readily exchanges guanine nucleotides at its "exchangeable" site and one (approximately 20% of the total) which exchanges nucleotides poorly. Those dimers that exchange GTP readily in Mg^{2+}-containing buffers appear to be the same dimers that rapidly release bound nucleotide in Mg^{2+}-free buffers.

The nature of the interaction between guanine nucleotides (GXP)[1] and tubulin is still not fully understood (Jacobs, 1979; Timasheff & Grisham, 1980). It is well established that two molecules of nucleotide (either GDP or GTP) are tightly bound to tubulin that has been isolated in the presence of GTP (Weisenberg et al., 1968; Berry & Shelanski, 1972). The binding sites have been distinguished on the basis of their ability to bind [^{3}H]GTP when incubated in vitro for short intervals. The nonexchangeable site (N site) probably contains GTP that never exchanges for free nucleotide in vitro and that can be released from that site only when the protein is denatured (Spiegelman et al., 1977). The exchangeable site (E site) contains a molecule of either GDP or GTP which is thought to exchange rapidly with free nucleotide in vitro (Weisenberg et al., 1968). During polymerization of tubulin, GTP at the E site is hydrolyzed to GDP; this hydrolysis lags behind the incorporation of a dimer into a microtubule (Penningroth & Kirschner, 1977; Carlier & Pantaloni, 1981). In order to understand better the role of GTP binding and hydrolysis during the assembly of microtubules, it is helpful first to understand the nature of the binding equilibrium between the nucleotide and the E site of tubulin. Approaches to studying this binding at equilibrium have been complicated because the dissociation constant for the E-site nucleotide is apparently so small (Zeeberg & Caplow, 1979) that tubulin is always isolated with nucleotide bound at both sites and because tubulin is very unstable when the E site is empty (Weisenberg et al., 1968; Arai et al., 1975). Hence, equilibrium conditions have never been applied to produce a straightforward binding isotherm. The difficulty of obtaining complete dissociation of the E-site GXP has led to the use of charcoal (Penningroth & Kirschner, 1977; Sandoval et al., 1977; Maccioni & Seeds, 1977, 1982), phosphatases (Purich & MacNeal, 1978), and a coupled enzyme system (MacNeal & Purich, 1978a; Brylawski & Caplow, 1983) to remove nucleotides completely or to ensure full exchange of one nucleotide for another. The apparent instability of tubulin has led investigators to measure equilibrium constants by methods that are rapid. These methods do not allow one to ascertain

† This work was supported by Research Grant GM25638 and Training Grant GM07319 from the National Institutes of Health, by a Mellon Foundation Regional Faculty Development Grant to Vanderbilt University (H.B.C.), and by grants from the Vanderbilt University Research Council and the Vanderbilt University Natural Sciences Committee.

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[1] Abbreviations: A_x, absorbance at x nm; CD, circular dichroism; CD buffer, 0.01 M Pipes–NaOH (pH 6.9), 0.1 M NaCl, 1 mM $MgSO_4$, 2 mM EGTA, and 0.2 mM DTE; DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GXP, guanine nucleotide; MAPs, microtubule-associated proteins; *n*XMT, microtubule protein purified through *n* cycles of in vitro assembly and disassembly; $NaDodSO_4$, sodium dodecyl sulfate; PCA, perchloric acid; PC-tubulin, tubulin purified by phosphocellulose chromatography; PD buffer, 0.1 M Pipes–NaOH (pH 6.9) and 2 mM DTE; PMD buffer, 0.1 M Pipes–NaOH (pH 6.9), 1 mM $MgSO_4$, 2 mM EGTA, and 2 mM DTE; Pipes, 1,4-piperazinediethanesulfonic acid; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid.

0006-2960/85/0424-0768$01.50/0

easily whether equilibrium has been attained (Zeeberg & Caplow, 1979; Jacobs & Caplow, 1976; Levi et al., 1974; Maccioni & Seeds, 1977). The necessity of unusual methods for nucleotide removal and rapid techniques for measurement of equilibrium constants may have masked some underlying complexity in tubulin's nucleotide-binding behavior.

The notion that there is a single freely exchangeable site has been an underlying assumption in many studies of guanine nucleotide (GXP) binding. Numerous investigations have made use of radioactively labeled GTP and of analogues of GTP to study the effects of substitution of these nucleotides without first quantitatively verifying whether all or only a fraction of the E sites were saturated with the labeled nucleotide under conditions of presumed saturation. In these studies, anomalous GXP binding could have been overlooked.

It was demonstrated early that Mg^{2+} is required for assembly and stability of microtubules (Weisenberg, 1972; Olmsted & Borisy, 1973, 1975) and for stability of tubulin (Weisenberg et al., 1968; Arai et al., 1975; Himes et al., 1977). Consequently, studies of the binding and exchange of nucleotides by tubulin have been carried out in Mg^{2+}-containing buffers. Recent evidence (Maccioni & Seeds, 1982; Jemiolo & Grisham, 1982) suggests that the nucleotide-binding process is affected by the presence of Mg^{2+}.

The results presented in this paper show that incubation of PC-tubulin in vitro with [^{3}H]GTP at concentrations from 70 μM to 1.0 mM at temperatures from 0 to 24 °C results in exchange of less than one GXP per tubulin dimer, such that the difference between the total GXP bound per tubulin and the [^{3}H]GTP bound per tubulin is consistently 1.2 ± 0.1. It is also demonstrated that when tubulin is gel filtered into a Mg^{2+}-free buffer, on two sequential columns, 0.4 mol of GXP is immediately lost per mol of tubulin without appreciable loss of the protein's ability to polymerize when both GTP and Mg^{2+} are added back. The nucleotide molecules that are released in this Mg^{2+}-free buffer are, within error, those which had exchanged with free [^{3}H]GTP upon prior incubation in vitro in the presence of Mg^{2+}. These results lead to the conclusion that tubulin may be heterogeneous with respect to its GXP binding behavior.

Experimental Procedures

Reagents. Colchicine, Pipes, EGTA, DTE, and GTP (type II S) were obtained from Sigma Chemical Co. [8-^{3}H]GTP (9.8 Ci/mmol), [8,5′-^{3}H]GTP (38.0 Ci/mmol), and [^{3}H]-colchicine (37.2 Ci/mmol) came from New England Nuclear. The GTP was dried under N_2 to remove possible contaminating tritiated water (Zeeberg & Caplow, 1979) and was then redissolved in a small amount of water or the appropriate buffer. ATP was purchased from P-L Biochemicals.

Experimental Conditions. All experiments were performed in PMD buffer [0.1 M Pipes–NaOH (pH 6.9), 2 mM EGTA, 1 mM $MgSO_4$, and 2 mM DTE] or in PD buffer [0.1 M Pipes–NaOH (pH 6.9) and 2 mM DTE] at 0–4 °C except where noted. Equilibration with buffers was achieved throughout by gel filtration of tubulin on Sephadex G-25 (Pharmacia).

Preparation of Brain Tubulin and MAPs. Microtubule protein (3XMT) was purified from bovine brain by the method of Shelanski et al. (1973) as modified by Berkowitz et al. (1977). In some preparations, 2.5 mM ATP was included in the first polymerization cycle to increase yields. Tubulin was separated from microtubule-associated proteins (MAPs) by chromatography of the 3XMT on Whatman P11 phosphocellulose (Weingarten et al., 1975; Detrich & Williams, 1978). Both the purified tubulin (PC-tubulin) and MAPs were frozen at −196 °C in PMD containing 0.1 mM GTP, and, in the case of MAPs, 0.7 M NaCl, and stored at −75 °C. Prior to use in polymerization studies, MAPs were thawed, centrifuged for 60 min at 95500*g* to sediment small amounts of contaminating neurofilaments, concentrated to approximately 10 mg/mL in an Amicon pressure cell fitted with a PM 30 Diaflo membrane, and gel filtered through Sephadex G-25 in PMD buffer. The solution of MAPs, thus processed, was frozen by dropping into liquid nitrogen at −196 °C and then stored at −75 °C. Immediately prior to an experiment, tubulin and processed MAPs were thawed and centrifuged at 1000*g* for 10 min. Tubulin was then equilibrated in the appropriate buffer on a Sephadex G-25 column prior to each experiment.

PC-tubulin was used within 50 days of preparation, and the purity of each preparation was checked by $NaDodSO_4$ electrophoresis on heavily overloaded $NaDodSO_4$ gels (Laemmli, 1970), as described by Detrich & Williams (1978). MAPs were found to retain their ability to stimulate polymerization of tubulin up to 6 months after their preparation.

Protein and Nucleotide Determinations. Protein concentrations were determined as described by Detrich & Williams (1978) by the method of Bradford (1976) and by the absorbance at 278.5 nm in nucleotide-free buffer. An extinction coefficient of 1.23 mL mg^{-1} cm^{-1} was employed (Detrich & Williams, 1978). A molecular weight of 100 000 (Krauhs et al., 1981; Ponstingl et al., 1981; Valenzuela et al., 1981) was used for tubulin. Guanine nucleotide (GXP) bound to tubulin was assayed by precipitating tubulin in PMD buffer (DTE concentration reduced to 0.2 mM). Exactly 0.1 volume of 5.5 N perchloric acid (PCA) was added, and the solution was incubated at 0 °C for a minimum of 30 min, followed by centrifugation for 30 min at 1000*g*. The concentration of DTE was lowered to avoid an uncertainty in the absorbance of PCA extracts that arises from differential oxidation of DTE. (Oxidized DTE displays a broad absorption peak at 275 nm.) The concentration of GXP was calculated from the absorbance of the extracts at 256 nm when read against PMD (0.2 mM DTE) buffer, similarly extracted with PCA. A molar extinction coefficient of 1.24×10^4 M^{-1} cm^{-1} was employed for GXP. To ensure that the extracts were not contaminated by other absorbing species, only those which displayed a normal GXP spectrum from 310 to 240 nm with a ratio of A_{280} to A_{256} of 0.66 ± 0.01 were used.

Guanine Nucleotide Binding Assay. The number of molecules of GXP bound to each tubulin dimer was determined by gel filtration of Sephadex G-25 or by column centrifugation using Sephadex G-50 (Penefsky, 1977). For the gel filtration studies, tubulin samples of 0.5–2.0 mL and of protein concentration 1–10 mg/mL were applied to a 0.9 × 25 cm column equilibrated in PMD buffer with 0.2 mM DTE. The peak fraction eluted from this column was assayed for protein both by the Bradford assay and by UV absorbance and was also assayed for guanine nucleotide content as described above. The protein concentration inferred from the absorbance was used in all final calculations of the number of bound GXPs per tubulin. When [^{3}H]GTP was present, the peak fraction was assayed for protein, and each of two 0.5-mL aliquots was counted in 10 mL of ACS scintillant (Amersham/Searle) on a Beckman Model LS-7000 scintillation spectrometer. Count rates were reduced to disintegration rates by employing channels ratio quench correction. The column centrifugation data were corrected for the counts when only [^{3}H]GTP (no tubulin) was applied to the column. This was typically less than 1% of the counts observed with tubulin + [^{3}H]GTP. The specific activity of the [^{3}H]GTP employed in the exchange

experiments was corrected for nonradioactive GTP released from tubulin during exchange according to the following equation:

$$\text{specific activity} = \frac{x}{y + zT}$$

where x is microcuries of [^{3}H]GTP, y is micromoles of GTP, and T is the micromoles of tubulin in the incubation mixture. z, the moles of guanine nucleotide exchanged per mole of tubulin, was calculated by taking the limit for an infinite number of successive approximations by the formula

$$z = \frac{Hy}{(x - H)T}$$

where H is the number of microcuries bound by T.

The purity of [^{3}H]GTP was determined by thin-layer chromatography on poly(ethylenamine)-impregnated cellulose (Cel 300 PEI/UV, Brinkmann) using a 1 M formic acid–0.25 M LiCl solvent and by high-performance liquid chromatography according to the method of Axelson et al. (1981). The purity at the time of purchase of both labels employed, after drying and resuspension, was 98%. No correction for this impurity has been made, no results on samples stored more than 1 year are reported, and the results in Table II reflect samples of 98% purity.

Colchicine Binding Assay. The gel filtration technique (Weisenberg et al., 1968; Wilson et al., 1974) was used to assay for colchicine binding. One-milliliter aliquots of tubulin were mixed at 0 °C with 0.1 mL of ^{3}H-labeled 1 mM colchicine (10 μCi/mL) and incubated at 37 °C for 20 min, gel filtered, then assayed for protein, and counted for [^{3}H]colchicine content as described above.

Analytical Ultracentrifugation. Sedimentation velocity studies were performed with a Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference optics and a helium–neon laser light source (Williams, 1972). The experiments were performed at 44 000 or 48 000 rpm at 4 °C in cells with double-sector aluminum-filled Epon centerpieces of 12- or 30-mm optical path and sapphire windows. A blank run, with the appropriate buffer as sample, was performed with each experimental run. Sedimentation coefficients were calculated from plots of the natural logarithm of the square root of the second moment of the interference fringe boundary (Schachman, 1959) vs. time, and corrected to 20 °C and water. For patterns that displayed broad heterogeneous boundaries, the distribution of sedimentation coefficients, $g(s)$, vs. the sedimentation coefficient, was evaluated by application of the Bridgemann equation (Bridgeman, 1942; Fujita, 1975):

$$g(s) = (1/c_0)(dc_0/ds)$$

where c_0 is the initial concentration. Values of $g(s)$ were extrapolated to infinite time, and both $g(s)$ and s were corrected to 20 °C and water. The tubulin heterodimer was considered a marker in these distributions, and its peak position was adjusted to $(5.4–5.6)s_{20,w}$ (derived from second moment calculations) to correct for small uncertainties in the determination of the time of centrifugation and the meniscus position.

Polymerizations. Microtubules were assembled in a thermojacketed cuvette holder in a Cary 118 spectrophotometer by raising the temperature from 10 to 36 °C. The polymerization buffer was PMD containing 1 mM GTP and 1 g of MAPs per 2 g of PC-tubulin. The absorbance at 350 nm was monitored until a plateau was reached. Microtubules were then disassembled by lowering the temperature to 10 °C and maintaining that temperature until no further change occurred. The difference in maximum absorbance and the absorbance after depolymerization was recorded as ΔA_{350}.

Circular Dichroism Spectroscopy. CD spectra were recorded at 26 °C on a Cary 60 spectropolarimeter equipped with a CD attachment. A cylindrical cell of 0.5-mm path length was employed. A full-scale sensitivity of either 40×10^{-3} or 100×10^{-3} deg and a time constant of 3 s were used. Duplicate scans were made of all sample and blank solutions, and reproducibility was invariably within $\pm 1 \times 10^{-3}$ deg. A value of 110 was used for the mean residue weight for tubulin. To obtain readings at wavelengths shorter than 220 nm, the PMD buffer was altered so that less Pipes and less DTE were present but the ionic strength was approximately the same. The CD buffer contained 0.01 M Pipes–NaOH (pH 6.9), 0.1 M NaCl, 1 mM $MgSO_4$, 2 mM EGTA, and 0.2 mM DTE.

Results

Effects of Mg^{2+} upon the Binding of Nucleotides to Tubulin. To pursue the effect of Mg^{2+} upon the nucleotide–tubulin interaction, we equilibrated PC-tubulin with Mg^{2+}-free buffer by gel filtration. Gel filtration took less than 15 min. Twenty-two minutes after the beginning of gel filtration, 1 mM EDTA was added to half of the tubulin. The GXP/tubulin ratio and the extent of ability to polymerize were determined at fixed intervals for both aliquots. As indicated in Figure 1, the initial equilibration with Mg^{2+}-free buffer reduced the number of bound nucleotides per tubulin dimer from 1.7 ± 0.1 to 1.3 ± 0.2. When both $MgSO_4$ and GTP were added back to a final concentration of 1 mM each, this tubulin polymerized to nearly the same extent as the control, which was never deprived of Mg^{2+} or GTP, and after gel filtration into Mg^{2+}-free buffer (PM buffer), it was found to have 1.7 ± 0.1 bound GXP per tubulin dimer, indicating that no change in GXP binding ability resulted from the short incubation in Mg^{2+}-free buffer. Upon prolonged incubation in the Mg^{2+}-free buffer, with or without EDTA, the GXP/tubulin ratio drops exponentially. Numerous control incubations of PC-tubulin in PMD buffer at 4 °C showed less than 5% loss of the ability to polymerize in 4–5 h. The ability to polymerize when 1 mM $MgSO_4$ and 1 mM GTP are added falls at approximately the same rate as does the GXP/tubulin ratio, indicating that this release, unlike the initial release, reflects the irreversible denaturation of the molecule. When incubated for 30 min at 0 or 23 °C in PMD with 1 mM GTP, tubulin which had previously been incubated for 2.5 h in Mg^{2+}-free buffer rebound 0.35 mol/mol of tubulin, increasing its bound nucleotide content from 1.07 to 1.42 GXP/tubulin. Control tubulin maintained in PMD buffer dropped from 1.7 to 1.6 mol of bound GXP in 2.5 h and showed no increase upon 30-min incubation with 1 mM GTP in PMD. It appears that tubulin denatures with a half-life of approximately 8 h in Mg^{2+}-free buffer and with a half-life of 4 h in the same buffer with 1 mM EDTA and that a proportion of the tubulin retains the ability to bind GTP and to polymerize when Mg^{2+} and GTP are added back to the buffer.

CD Spectra of Tubulin in Mg^{2+}-Free Buffers. To measure the effect of incubation in Mg^{2+}-free buffer with and without EDTA upon the secondary structure of tubulin, the molecule was exposed to each treatment, as described in the legend to Figure 2. Exposure for 100 min to a buffer with no Mg^{2+} resulted in very little change in the CD spectrum from 210 to 260 nm, but upon prolonged incubation (5.5 h) in a buffer with no Mg^{2+}, a decrease in molar ellipticity was observed. This apparent loss of secondary structure was slightly accelerated when 1 mM EDTA was included for 3 h of the incu-

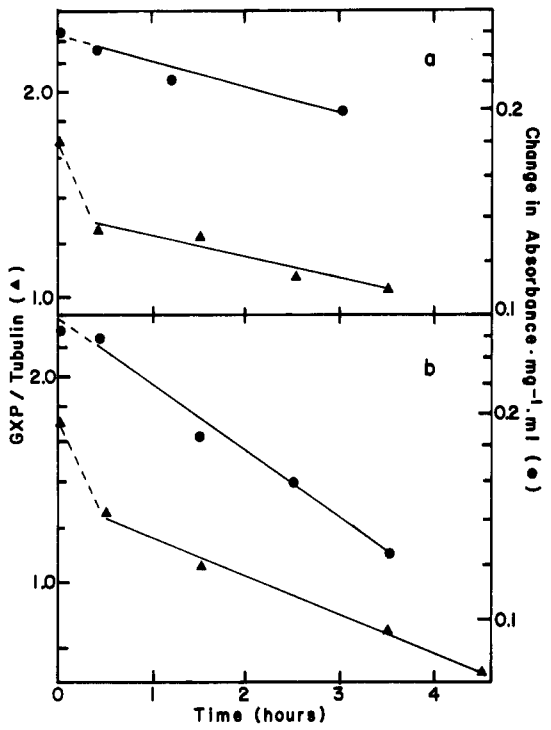


FIGURE 1: Effect of incubating tubulin in Mg^{2+}-free buffer upon the ratio of bound GXP and upon its ability to polymerize. Tubulin, placed on Sephadex G-25 in PD buffer at time zero, was eluted within 10 min and divided into two halves. (a) One half was incubated at 3 mg/mL, and separate aliquots were assayed at timed intervals for GXP/tubulin (left ordinate; ▲) after a second filtration through Sephadex G-25 in PD (0.2 mM DTE) and for ability to polymerize (right ordinate; ●) after the buffer was adjusted to PMD containing 1 mM GTP and MAPs (1 g of MAPs per 2 g of tubulin) as described under Experimental Procedures. (b) To the second half was added 1 mM EDTA 22 min after the first Sephadex column. Aliquots of the tubulin (2.9 mg/mL) were removed for assay of GXP/tubulin and for ability to polymerize. Symbols as above. (The GXP/tubulin ratio at time zero was determined on control samples of tubulin equilibrated on Sephadex G-25 in PMD buffer. The polymerization at time zero was determined on a control sample of tubulin never exposed to PD buffer as described under Experimental Procedures. The dashed lines represent changes that presumably occurred during the processing of the samples for the 25-min point.)

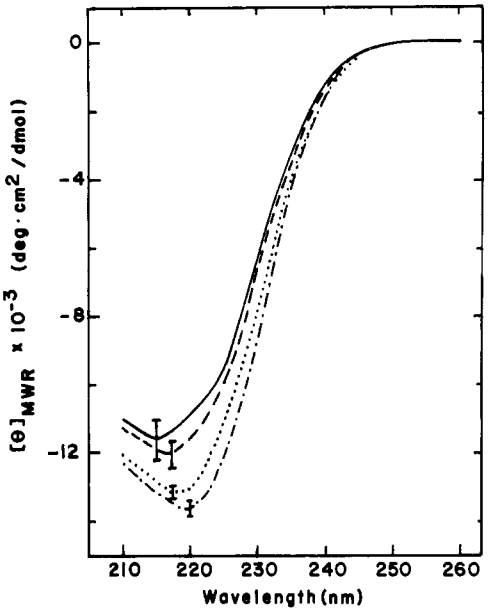


FIGURE 2: Circular dichroism of tubulin after incubation in Mg^{2+}-free buffers. (a) (–·–) Control in buffer with Mg^{2+}. Tubulin was equilibrated with CD buffer (0.01 M Pipes–NaOH, 0.1 M NaCl, 1 mM $MgSO_4$, 2 mM EGTA, and 0.2 mM DTE) and allowed to stand 120 min at 4 °C before CD spectroscopy. (b) (···) 100 min in Mg^{2+}-free CD buffer. Tubulin was equilibrated with CD buffer lacking both $MgSO_4$ and EGTA and allowed to stand 180 min at 4 °C before CD spectroscopy. (c) (– – –) 250 min in Mg^{2+}-free buffer + 80 min in Mg^{2+}-free CD buffer. Tubulin was incubated in PD buffer for 250 min and then equilibrated in CD buffer with $MgSO_4$ and EGTA omitted. It stood 80 min at 4 °C in this buffer before CD spectroscopy. (d) (—) 210 min in Mg^{2+}-free buffer with EDTA + 100 min in Mg^{2+}-free CD buffer. Tubulin was incubated in 0.1 M Pipes (pH 6.9) with 2 mM DTE and 1 mM EDTA for 210 min and then equilibrated in CD buffer lacking $MgSO_4$ and EGTA. It stood in this buffer 100 min at 4 °C before CD spectroscopy.

bation time. Separate CD experiments done in CD buffer ± 1.0 mM Mg^{2+} and 2.0 mM EGTA and ± 0.1 mM GTP as a function of time revealed a comparable time-dependent loss of secondary structure under all conditions (data not shown). The presence of GTP in the buffer (±Mg^{2+}–EGTA) affords only marginal protection of secondary structure compared to –GTP conditions. These data would support the interpretation that, in a buffer containing no Mg^{2+}, tubulin releases approximately 0.4 mol of nucleotide rapidly and reversibly. This release is accompanied by no major conformational change. Upon subsequent incubation, additional nucleotide is released as more tubulin denatures irreversibly with concomitant loss of secondary structure. The addition of 1 mM EDTA to the buffer appears to accelerate slightly the rate of denaturation.

Sedimentation Velocity of Tubulin in Buffer with No Mg^{2+}. Figure 3 presents curves of $g(s_{20,w})$ vs. $s_{20,w}$ derived from sedimentation velocity experiments on PC-tubulin solutions exposed to Mg^{2+}-free buffer (PD) with and without EDTA. Here, $s_{20,w}$ is the sedimentation coefficient, and $g(s_{20,w})$ is the normalized fraction of solute having that sedimentation coefficient. These are compared to curves obtained with PC-tubulin in Mg^{2+}-containing buffer (PMD) with 0.1 mM GTP. Nearly all of the protein in all three cases sedimented as a single species with an $s_{20,w}$ = 5.4–5.6 S. This sedimentation behavior is typical of PC-tubulin prepared as described in this paper (Williams & Detrich, 1979). In addition, both Mg^{2+}-free solutions contained a small amount of protein sedimenting as a broad heterogeneous boundary. The relative amount of material in this heterogeneous boundary increased with longer times of exposure of the protein to Mg^{2+}-free buffer. The presence of EDTA appeared to have no appreciable effect on the distribution. This broad zone appears to reflect a small population of tubulin that has irreversibly aggregated. Because this material appears at relatively late times (cf. Figure 1), it would be consistent with the nucleotide binding and polymerization data to assert that the small amount of observed aggregation accompanies the irreversible loss of at least one GXP. The major conclusion from these data is that tubulin is predominantly present as the heterodimer after 4–5 h of incubation in PD buffer.

Exchange of [^{3}H]GTP with PC-Tubulin. In order to determine whether the nucleotide which is removed from tubulin in a Mg^{2+}-free buffer is released from the exchangeable site, we first incubated PC-tubulin with [^{3}H]GTP to saturate the E site. Figure 4 shows the results obtained when tubulin was incubated with 0.1 mM [^{3}H]GTP for 2 h at each of three temperatures and was assayed at intervals for the ratio of bound [^{3}H]GTP per tubulin dimer. The maximum ratio obtained was 0.56. This ratio remained constant for 2 h at 0 °C but decreased in a roughly exponential fashion at both 24 and 37 °C, probably reflecting the instability of the tubulin molecule at these temperatures. This same preparation of tubulin bound 0.84 mol of colchicine per dimer when incubated with the drug at 37 °C for 90 min. Thus, it appears that not every protein molecule capable of binding colchicine can un-

Table I: Release of Bound Nucleotide from Tubulin during Incubation in a Magnesium-Free Buffer[a]

time (min)	buffer	GXP/tubulin	change in GXP/tubulin	[^{3}H]GTP/tubulin	change in [^{3}H]GTP/tubulin
0	PMD	1.82		0.60	
0	PD	1.70	0.12	0.47	0.13
60	PMD	1.77	0.05	0.48	0.12
60	PD	1.41	0.41	0.23	0.37

[a] Tubulin (6.2 mg/mL) was incubated in PMD at 23 °C for 30 min with 0.128 mM [^{3}H]GTP to label the exchangeable (E-site) nucleotide binding site and was divided into two halves. All subsequent incubations and gel filtrations were at 0 and 4 °C. At 0 min, half was gel filtered into PMD buffer, and the other half was gel filtered into PD buffer to remove unbound nucleotides. Aliquots of the peak fractions were assayed for total nucleotides (GXP) per tubulin and for [^{3}H]GTP per tubulin. Other aliquots were allowed to stand in their respective buffers until 60 min had elapsed and were gel filtered into their respective buffers to remove nucleotides released from tubulin during this interval. The peak fractions were assayed as before for GXP/tubulin and for [^{3}H]GTP/tubulin. The changes in GXP/tubulin and in [^{3}H]GTP/tubulin were calculated by subtracting the value at each interval in either buffer from that in PMD buffer at 0 min.

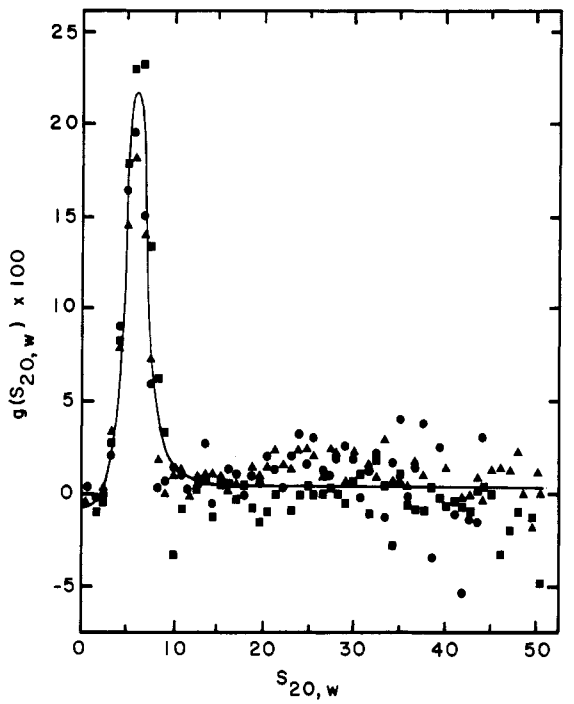


FIGURE 3: $g(s_{20,w})$ vs. $s_{20,w}$ derived from sedimentation velocity experiments with PC-tubulin in Mg-free buffers sedimented at 48 000 rpm. Concurrent measurements of GXP/tubulin ratios on separate aliquots of solutions (●, ▲) gave values of 1.0–1.1. It must be noted that the magnitude of $g(s)$, and thus the total area under the curves, was normalized by the loading concentration as estimated by the Bradford dye binding assay. An alternate method to estimate C_0 would be to count the fringes across all boundaries during centrifugation, correcting for radial dilution. However, this would neglect rapidly sedimenting material >100 S. Such flocculant material was often visually apparent under extreme solution conditions (H. B. Croom et al., unpublished results). Consequently, the distributions have not been normalized to give total areas of unity [i.e., $\int g(s)ds = 1$]. These data indicate a small relative shift of tubulin into more rapidly sedimenting species. The curve indicates the trend of the data. (■) Control: PC-tubulin (1.51 mg/mL) in PM buffer + 0.1 mM GTP; (●) PC-tubulin (2.14 mg/mL) incubated in PD buffer + 1 mM EDTA for 90 min prior to the start of centrifugation; (▲) PC-tubulin (2.14 mg/mL) incubated in PD buffer for 4 h prior to centrifugation.

dergo an exchange of one of its bound GXP molecules for GTP in the medium. This observation indicates that the nucleotide binding sites of PC-tubulin are not correctly described by assuming that each tubulin dimer has one nonexchangeable and one exchangeable site (Weisenberg et al., 1968) and that all exchangeable sites are identical. The fraction of poorly exchangeable E-site GXP (TB′ in Scheme I; see Discussion) cannot be attributed to the presence of oligomers in the present case, since essentially all of the tubulin is present as the 5.8 S dimer (cf. Figure 3). Such an explanation was invoked in the case of microtubule protein by Weisenberg et al. (1976)

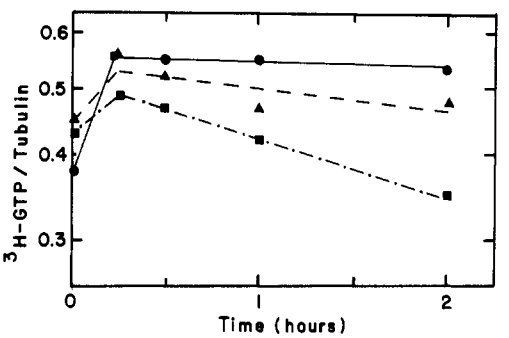


FIGURE 4: Kinetics of [^{3}H]GTP exchange at three temperatures. Tubulin in PMD was divided into three parts and preincubated at the designated temperatures for 15 min. At time zero, [^{3}H]GTP (10.7 μCi/μmol) was added to yield a final concentration of 80.2 μM [^{3}H]GTP with a tubulin concentration of 28.8 μM in each. At intervals, aliquots were assayed for [^{3}H]GTP/tubulin. The specific activity of GTP was approximated as described under Experimental Procedures for time points at 0 and 15 min. For later points, the specific activity calculated at time 15 min was used: (●) 0 °C; (▲) 24 °C; (■) 37 °C.

and by Jacobs & Huitorel (1979).

Table I reports the results of an experiment in which tubulin was labeled with [^{3}H]GTP prior to gel filtration into nucleotide-free buffers with or without Mg^{2+}. Immediately after filtration into the Mg^{2+}-free buffer, tubulin had released a total of 0.12 GXP per dimer, all of which was [^{3}H]GTP. After a 60-min incubation in the same buffer at 0 °C, tubulin had released 0.41 GXP per dimer of which 0.37 per dimer was [^{3}H]GTP. During the same incubation in Mg^{2+}-containing buffer, almost no GXP or [^{3}H]GTP was released from the dimer as would be expected from the small dissociation constant of E-site GXP. It appears that the guanine nucleotide released in a Mg^{2+}-free buffer during the first 60 min comes from the population of sites which also exchanges with [^{3}H]GTP in vitro. The simplest explanation of this result is to assume that there are two classes of E sites. One class exchanges nucleotide rapidly in the presence of Mg^{2+} and releases it preferentially in the absence of Mg^{2+}. The other class neither exchanges nor releases GXP rapidly in the absence of Mg^{2+}.

Additional exchange experiments were performed to investigate the possibility that the results above are due to the low [^{3}H]GTP concentrations used or to the gel filtration binding assay employed. Table II shows results at two concentrations of GTP (0.1 and 1.0 mM) and at two temperatures (0 and 23 °C) using two different binding assays, a slow gel filtration technique and a more rapid column centrifugation technique (Penefsky, 1977). Under all of these conditions, the difference between the total GXP bound per dimer and the [^{3}H]GTP exchanged per dimer is consistently 1.20 ± 0.1. These data strongly support the notion that PC-tubulin consists

Table II: Exchange of [^{3}H]GTP for Nucleotides Bound to Tubulin[a]

	[GTP] (mM)	incubation temp (°C)	GXP/tubulin (total)	[^{3}H]GTP/tubulin (exchanged)	Δ^b
gel filtered	0.1	0	1.78	0.63	1.15
		23	1.91	0.65	1.26
	1.0	0	1.94	0.77	1.17
		23	1.81	0.76	1.05
column centrifuged	0.1	0	2.02	0.72	1.30
		23	1.82	0.67	1.15
	1.0	0	2.00	0.71	1.29
		23	1.91	0.75	1.16

[a] Tubulin (3 mg/mL) was incubated in PMD for 30 min at the indicated temperatures and [^{3}H]GTP concentrations and then either gel filtered or column centrifuged (Penefsky, 1977) to remove unbound GTP. [b] The difference between GXP/tubulin and [^{3}H]GTP/tubulin is recorded as Δ. The [^{3}H]GTP used was [8-^{3}H]GTP, 98% pure in the experiments involving gel filtration, and [8,5′-^{3}H]GTP, 98% pure in the experiments involving column centrifugation. The use of singly or doubly labeled GTP has been confirmed to have no effect on the results of these experiments.[2]

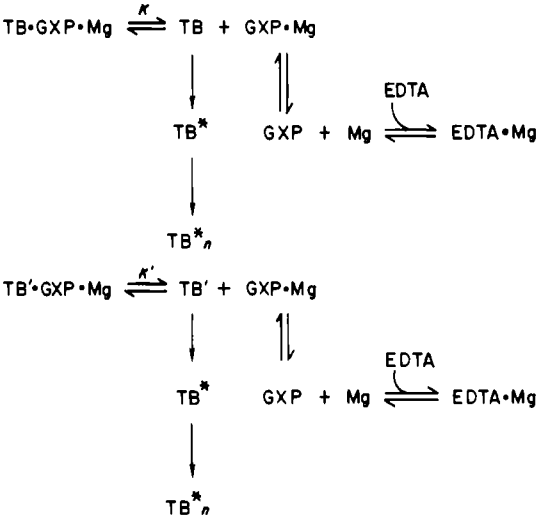
of two populations with respect to the ability of exchange [^{3}H]GTP in vitro.

Discussion

Numerous studies (Olmsted & Borisy, 1975; Lee & Timasheff, 1977; Himes et al., 1977) indicate that one Mg^{2+} is bound with high affinity to each tubulin dimer and that the presence of Mg^{2+} in excess of this amount is required for assembly of microtubules. It is also known that manganese and a number of other metals will replace magnesium at a high-affinity site (Buttlaire et al., 1980; MacNeal & Purich, 1978a,b). Magnesium ion also appears to be intimately involved in the binding of nucleotides by tubulin (Jacobs, 1979). Recent results of Maccioni & Seeds (1982) have shown that larger amounts of GXP can be removed from tubulin by charcoal extraction in the absence of Mg^{2+} than in its presence. Jemiolo & Grisham (1982) found that removal of the exchangeable nucleotide by treatment with alkaline phosphatase attached to agarose results in the loss of the high-affinity manganese binding site. Thus, it appears that it is a metal–nucleotide complex that binds at the exchangeable nucleotide binding site. The ready removal of a fraction of E-site GXP from tubulin under Mg^{2+}-depleted conditions must reflect the dissociation of this complex from a fraction of the tubulin molecules.

The present evidence indicates that PC-tubulin may be heterogeneous not only with respect to binding of the nucleotide–magnesium complex but also with respect to nucleotide exchange. The evidence consists of the following three findings. (1) A fraction equal to 0.4 ± 0.2 GXP per tubulin dimer is rapidly released at early times in Mg^{2+}-free buffer. (2) A fraction of tubulin will exchange externally added [^{3}H]GTP. (3) The same molecules that rapidly exchanged GTP are the ones that release their GTP upon subsequent incubation in Mg^{2+}-free conditions. This heterogeneity is not the consequence of a large conformational rearrangement of the tubulin, since neither the colchicine binding, the ability to polymerize, the sedimentation velocity pattern, nor the circular dichroic properties of the molecule are extensively changed by short exposure to Mg^{2+}-free conditions. This evidence is at variance with the notion that all tubulin molecules have an equivalent exchangeable site, but it is not (as well shall show in the following discussion) inconsistent with the overwhelming bulk of the data concerning nucleotide exchange by tubulin. Scheme I, a working hypothesis, can explain the experimental observations. The indicated species GXP·Mg represents either a nucleotide–metal ion complex or an interdependence (as might be mediated by a protein conformational change) between the binding of GXP and the binding of Mg^{2+}. The tubulin species (TB and TB′) are presumed to have GXP at the N site. TB* may or may not

Scheme I



have N-site GXP. GXP·Mg can bind at the E site of TB or TB′. The species TB′ binds nucleotide very tightly and releases it very slowly, while TB binds GXP less tightly and releases it more rapidly. The dissociation constant K is larger than K'. Under our conditions of observation, the interconversion of TB and TB′ either is extremely slow or does not occur. Upon release of GXP·Mg, TB and TB′ become susceptible to irreversible conversion to TB*, the nonassembling species observed upon prolonged incubation in Mg^{2+}-free buffer. TB* can aggregate to form the precipitates observed in denatured tubulin.

When the protein is placed in Mg^{2+}-free buffer, substantial amounts of TB are formed from TB·GXP·Mg, but relatively little TB′·GXP·Mg is converted to TB′. This accounts for the observation that about 0.4 mol of GXP is released rapidly (Figure 1). The loss of nucleotide upon incubation in PD buffer in the presence of 1 mM GTP is similar to the very small loss in the control incubation in PMD buffer. This is consistent with the concentration dependence expected for a dissociating ligand. Subsequently, conversion from TB to TB* takes place. When both GTP and Mg^{2+} are restored to a solution of TB, it retains the ability to polymerize. However, TB*, having denatured, no longer polymerizes under these conditions. The rate of this denaturation is evidently increased when EDTA is added to the buffer, a fact that is explained by the dissociation of TB·GXP·Mg to give TB + GXP + EDTA·Mg.

In a recent study with PC-tubulin, Maccioni & Seeds (1982) found that 1.3 mol of GXP remained on the tubulin dimer after charcoal extraction and that 0.6 mol was easily removed. (These values have been corrected to reflect the true molecular weight of the tubulin heterodimer. In making comparisons

of other data with the data reported or cited here, a similar correction must be applied.) The fraction that retained its GXP appeared to be the fraction that polymerized in subsequent experiments. Such a species would correspond to TB′·GXP·Mg in the scheme above. These authors also found, by direct measurement of protein and nucleotide, that although only 0.6 mol of GXP per tubulin dimer was removed by charcoal treatment in the presence of 1 mM Mg^{2+}, 0.9 mol was removed by charcoal treatment in the presence of EDTA. If K' is small enough, the combined use of charcoal and EDTA might be necessary to shift the equilibrium substantially to the right. (Charcoal treatment may remove GXP from more than one site.) Buttlaire et al. (1980) reported the existence of two populations of tubulin-bound Mg^{2+}: one which exchanges readily with Mn^{2+} (about 70% of tubulin) and one which does not (about 30%). These two species might also be represented by TB·GXP·Mg and TB′·GXP·Mg. It has also recently been reported that there are two pools of tubulin, one pool that nucleates or elongates and one pool that can only elongate (Keates et al., 1983). Finally, numerous workers (Arai & Kaziro, 1976; Caplow & Zeeberg, 1980; Jacobs et al., 1974; Kirsch & Yarbrough, 1981; Maccioni & Seeds, 1982; Penningroth & Kirschner, 1977, 1978; Zeeberg & Caplow, 1979; Nath et al., 1983) have reported difficulties in removing or fully exchanging the "exchangeable" nucleotide quantitat ively. In fact, in the initial study of GXP binding to tubulin, Weisenberg et al. (1968) found that approximately 1.5 GXP (after correction for the molecular weight of tubulin) could be bound per tubulin dimer, of which about half was exchangeable. Timasheff and co-workers (S.N. Timasheff and O. Monasterio, personal communication) have also observed that only about 0.6 mol of nucleotide exchanges per mol of tubulin under ordinary conditions.

The molecular basis for the two putative functional forms of tubulin, TB and TB′, could reside in differences in amino acid sequence or in differences arising posttranslationally (e.g., phosphorylation, glycosylation, or disulfide formation). The existence of numerous "isotubulins" demonstrated by Lee and co-workers (George et al., 1981) and the presence of numerous tubulin genes [reviewed by Cleveland (1983)] certainly allow room for both possibilities. It is thus possible that different separation methods could yield different ratios of TB to TB′. In addition, there is evidence of different conformational forms of tubulin, potentially related to conformational transitions within the heterodimer–nucleotide complex prior to elongation (Carlier, 1983). Hamel & Lin (1981) have suggested that tubulin contains "two mutually exclusive, perhaps overlapping, sites, one with high affinity for GTP, the other with high affinity for GDP", based upon differences in GTP and GDP analogue inhibitory effects. This is consistent with conformational transitions in tubulin induced by changes in the nucleotide content of the heterodimer. Furthermore, the original work on the structure of the tubulin dimer by Ludueña et al. (1975, 1977) could not exclude the possibility of some of the soluble tubulin being in the form of homodimers, although it is not clear if their data would support 20–30% homodimers.

If the putative TB and TB′ are present, it seems likely that they may be interconvertible, at least in their GXP binding characteristics. One strong possibility is that they interconvert during assembly or disassembly of microtubules. It is generally believed that either a preincubation with GTP at 37 °C (Carlier, 1983) or an assembly–disassembly cycle in the presence of GXP (Caplow & Zeeberg, 1980; Weisenberg et al., 1976) will lead to exchange of 1.0 mol of nucleotide per mol of tubulin. If so, the completeness of exchange under these conditions may result from a rapid interconversion of TB and TB′ that could occur when tubulin is incorporated into a protofilament or microtubule. (However, preliminary experiments in our laboratory suggest that the ratio of TB′ or TB does not change during a cycle of polymerization–depolymerization. Polymerization of PC-tubulin with [^{3}H]GTP in 4 M glycerol incorporates labeled nucleotide to the same substoichiometric extent as a 30-min cold incubation (±4 M glycerol) with [^{3}H]GTP.[2]) Alternatively, the presence of other proteins might accelerate or retard GXP exchange. Among them might be copurifying molecules, akin to the exchange factor that facilitates exchange of GTP for GDP in the case of eukaryotic initiation factor 2 (Panniers & Henshaw, 1983). If that were the case, one would expect cruder preparations of microtubule protein to have a fraction of TB′ closer to zero than do more refined preparations of pure tubulin.

Other factors, as yet unrecognized, may affect the observed stoichiometry of binding of GXP to tubulin. Nothing is known about the binding of GXP to the individual dissociated (Detrich & Williams, 1978) α- and β-subunits of tubulin. An understanding of the role of dissociation of the dimer in GXP binding will require investigation of the dependence of the binding upon the concentration of both GXP and tubulin.[2] It is also possible that occupancy of the weak "third" nucleotide binding site (Zabrecky & Cole, 1980, 1982a,b; Maccioni & Seeds, 1982) may allosterically modulate the exchange of GTP at the sites observed here. Also, one cannot exclude categorically the possibility that the presence of TB′ is an artifact of the protein preparation method employed here, although the preliminary observations of Timasheff and co-workers mentioned above would argue that it may be a general property of tubulin, since they employed protein prepared by an independent method (Weisenberg et al., 1968; Lee et al., 1973). These possibilities are currently under investigation.

This paper argues strongly that there are present in tubulin solutions two kinds of dimers: one that exchange GTP rapidly and one that exchanges GTP with difficulty. Whether the difference between them is one of equilibrium constants or of rate constants, and what its structural basis might be, must await their isolation and further characterization.

ACKNOWLEDGMENTS

We thank Kate Welch for expert technical assistance, Cindy Young for excellent secretarial assistance, Fred Croom for pointing out the limiting form of the successive approximations, and David Puett for help with the circular dichroism experiments.

Registry No. GTP, 86-01-1; GDP, 146-91-8; Mg, 7439-95-4.

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