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Effect of Guanine Nucleotides on the Hydrophobic Interaction of Tubulin†

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Received July 5, 1989; Revised Manuscript Received February 12, 1990

ABSTRACT: The influence of guanine nucleotides on the binding of tubulin to hydrophobic components is investigated. Tubulin binds to a hydrophobic phenyl-Sepharose gel in a reversible, nucleotide-dependent way. Assembly-competent tubulin is released with ion-free water as eluent. It contains one guanosine triphosphate per dimer. More denatured tubulin needs a mixture of ethanol–water to elute. Consequently, hydrophobic interaction chromatography over phenyl-Sepharose represents an easy method for preparing polymerizable tubulin free of nucleotides at the exchangeable sites. While, in the absence of guanine nucleotide, the binding of tubulin to phenyl-Sepharose is rapid and immediately reversible on nucleotide addition, the binding of the nucleotide-dependent hydrophobic sites of tubulin to 1,8-ANS is slow, and its dissociation on nucleotide addition is poor. No differences are observed between the shielding of hydrophobic sites in the presence of GTP or GDP. Neither inorganic phosphate nor AlF_4^- is found to directly influence guanine nucleotides in their ability to shield hydrophobic sites.

Both α- and β-tubulin subunits are anionic polypeptides whose carboxyl-terminal sequences are particularly rich in acidic amino acids. These regions play an important regulatory role in the assembly of microtubules through the binding of microtubule-associated proteins (MAPs)[1] (Serrano et al., 1984; Littauer et al., 1986).

Less is known about the nature of the tubulin–tubulin interactions that lead to the formation of microtubules. An involvement of hydrophobic interactions can be argued by the fact that dimeric tubulin contains extensive and easily accessible regions capable of hydrophobic interaction (Andreu, 1982; Horowitz et al., 1984; Kocha et al., 1986; Prasad et al., 1987; Stephens, 1988). Association of strongly binding hydrophobic reagents inhibits self-assembly (Horowitz et al., 1984; Andreu & Timasheff, 1982). Also the increase of the enthalpy and entropy, and the negative heat capacity change accompanying the formation of microtubules (Lee & Timasheff, 1977; Hinz & Timasheff, 1986), indicates that the largest contribution of these thermodynamic properties is given by the removal of nonpolar protein surfaces from water.

Although the hydrophobic nature of proteins can be importantly influenced by their natural ligands (Desmet et al., 1987), up to now, no research on the effect of natural tubulin ligands, such as GTP and GDP, has been done. In the present study we clearly demonstrate that the removal of exchangeable guanine nucleotides strongly influences the hydrophobic behavior of tubulin and that assembly-competent tubulin, containing one guanosine triphosphate per dimer, can be obtained by hydrophobic interaction chromatography on phenyl-Sepharose. As microtubules are stabilized by inorganic phosphate and by its structural analogue AlF_4^- (Carlier et al., 1988), we also investigated whether these anions, together with guanine nucleotides, can modulate the hydrophobic behavior of tubulin.

Materials and Methods

Pig brains are obtained from freshly slaughtered animals and kept in ice-cold buffer containing 100 mM MES–KOH, 1 mM $MgSO_4$, and 1 mM EGTA, pH 6.5. The brains are used within 1 h after slaughter. The nucleotides GTP and GDP are purchased from Boehringer. 1,8-ANS is obtained from Molecular Probes and phenyl-Sepharose CL-4B from Pharmacia.

Preparation of Tubulin and Microtuble-Associated Proteins. Pig brain tubulin is prepared by two polymerization cycles, as described by Shelanski et al. (1973). The pellet obtained after the second polymerization is resuspended in a buffer containing 100 mM MES–KOH, 0.1 mM GTP, 1 mM $MgSO_4$, and 1 mM EGTA, pH 6.5, and stored in liquid nitrogen. Just before use, tubulin is purified from microtubule-associated proteins by chromatography on phosphocellulose. The elution buffer consists of MM buffer, containing in addition 40 mM KCl (or 15 mM K_2SO_4) and 0.05 mM GTP. The fraction of microtubule-associated proteins is eluted with 0.8 M NaCl and chromatographed over Sephadex G-25 with MM buffer containing 15 mM K_2SO_4. Protein concentrations are determined by the method of Folin–Ciocalteu, using bovine serum albumin as a standard. In order to calculate molar concentrations, the molecular weight of a tubulin dimer is taken to be 100 000.

† This work is part of Research Project 3.0063.86, supported by the Belgian "Fonds voor Geneeskundig Wetenschappelijk Onderzoek".

[1] Abbreviations: 1,8-ANS, 8-anilinonaphthalene-1-sulfonate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; MES, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAPs, microtubule-associated proteins; MM buffer, 10 mM MES–KOH (pH 6.5) and 1 mM $MgSO_4$; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PC-tubulin, phosphocellulose-purified tubulin.

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Hydrophobic Interaction Chromatography. Hydrophobic interaction chromatography is performed at 4 °C on a column containing fresh phenyl-Sepharose CL-4B. Columns of 1.1- and 1.6-cm diameter are used. Both columns are filled to a height of 5 cm, and the flow rate is 1 mL cm^{-2} min^{-1}. Before use, the gels are washed with 5 bed volumes of distilled water and equilibrated with 10 bed volumes of the first elution buffer. Before the sample is applied to the column, the salt concentration of the tubulin samples is adjusted to that of the first elution buffer, by adding a small volume of concentrated salt solution. During chromatography, the composition of the eluent is changed at the elution volumes indicated by the arrows on the chromatograms.

Guanine Nucleotide Determination. To analyze the nucleotide content in tubulin samples, the nucleotides are released from tubulin by precipitation of the protein in 2.5% (w/v) $HClO_4$. The suspension is incubated at 0 °C for 10 min, followed by centrifugation for 10 min at 1000*g*. The pH of the supernatant is adjusted to 3.0. The amount of guanine nucleotides is determined by HPLCchromatography on a μBondapak NH_2 anion-exchange column, 4 mm i.d. × 30 cm (Waters). A linear eluent system of 0.01 M $NH_4H_2PO_4$ (pH 3.0)–0.25 M $NH_4H_2PO_4$ (pH 5.0) is applied. Nucleotide concentrations are determined from peak areas obtained by UV detection at 254 nm.

Column Centrifugation. In an assay of the ability to rebind guanine nucleotides, tubulin is freed of unbound nucleotides by column centrifugation through 1-mL syringe columns filled with Sephadex G-50 Fine (Penefsky, 1977). The effluents (100 μL) of different columns are collected and used for determination of the amount of protein and guanine nucleotides as described above.

Microtubule Assembly Assay. The ability of tubulin to polymerize is checked by measuring the increase of the turbidity at 350 nm of a mixture of MAPs and tubulin in a Beckman spectrophotometer thermostated at 37 °C. The protein mixtures are made in MM buffer containing 15 mM K_2SO_4, 0.5 mM GTP, and 2.3 M glycerol.

Electron Microscopy. Small aliquots (10 μL) of the tubulin, polymerized as described above, are fixed in suspension at 37 °C by adding 10 μL of MM-buffered 0.5% glutaraldehyde. Following 30 s of fixation, single drops of the sample are deposited on formvar-coated grids for 30 s. Excess fluid is drawn off by using torn edges of filter paper. To each grid, one drop of cytochrome *c* (1 mg/mL MM buffer) is added during 15 s, followed by washing three times with a drop of distilled water. Excess water is drawn off with filter paper, and the samples are subsequently stained for 30 s with 1% (w/v) aqueous uranyl acetate. Finally, the uranyl acetate solution is removed with filter paper, and the grids are allowed to air-dry. Samples are examined by using a Zeiss EM 10 C transmission electron microscope operating at 60 kV.

SDS–PAGE. Fractions of tubulin desorbed from the phenyl-Sepharose column are characterized by electrophoresis on 7.5% polyacrylamide gels. The solutions for the preparation of the separating and stacking gels contain 6 M urea. Following electrophoresis, the protein bands are stained with Coomassie Brilliant Blue R and scanned in a Chromoscan 3 (Joyce-Loebl).

Fluorescence Measurements. All fluorescence measurements are made on an Aminco SPF-500 spectrofluorimeter, connected with a personal computer. The temperature is controlled by water circulating through the cell holder. Excitation occurs at 350 nm, and a 4-nm bandwidth is used. The emission is observed at 470 nm, and the bandwidth is 2 nm.

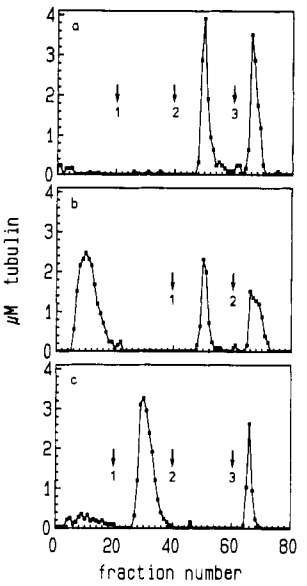

FIGURE 1: Hydrophobic interaction chromatography of tubulin on phenyl-Sepharose at 4 °C. Sample: 1 mL containing 1.6 mg of phosphocellulose-purified tubulin. Column: 1.1 × 5 cm. Flow rate: 1 mL/min. Fractions: 1 mL. (a) MM buffer containing 190 mM KCl is used for initial elution. It is changed to MM buffer containing 40 mM KCl at arrow 1, to water at arrow 2, and to a mixture of 50% (v/v) ethanol–water at arrow 3. (b) MM buffer containing 40 mM KCl is used for the initial elution. It is changed to water at arrow 1 and to a mixture of 50% (v/v) ethanol–water at arrow 2. (c) MM buffer containing 190 mM KCl is used for initial elution. It is changed to mM buffer containing 40 mM KCl and 0.5 mM GTP at arrow 1, to water at arrow 2, and to a mixture of 50% (v/v) ethanol–water at arrow 3. The tubulin content is determined as described under Materials and Methods.

Fluorescence intensities are corrected for inner-filter effects, due to the absorbance of 1,8-ANS at the excitation wavelength, according to the procedure of Mc Clure and Edelman (1967). The concentrations of 1,8-ANS in stock solutions are quantitated by using $\epsilon_M = 4.9 \times 10^3$ M^{-1} cm^{-1} at 350 nm.

Results

Effect of the Salt Concentration on the Interaction of Tubulin with Phenyl-Sepharose. In Figure 1a, the influence of the salt concentration on the adsorption and desorption of tubulin is demonstrated. When tubulin is applied in an MM buffer containing 190 mM KCl but no guanine nucleotides and eluted with the same buffer, practically all tubulin is retained on the phenyl-Sepharose gel. When the KCl concentration in the eluting MM buffer is decreased to 40 mM or even to 0 mM, no tubulin desorbs. One fraction of the tubulin elutes with water; another needs a 50% (v/v) ethanol–water mixture to desorb. With small columns (1.1 × 5 cm) and a high flow rate (1 mL/min), an average of 70% of the applied tubulin (1.6 mg) is recovered, indicating that 30% remained tightly bound. Longer columns, slower flow rates, and higher temperatures result in a smaller yield. The tubulin output is also strongly reduced when chromatographing over the more hydrophobic octyl-Sepharose gel, used by Prasad et al. (1987).

Figure 1b shows the effect of the KCl concentration. Tubulin applied in an MM buffer containing 40 mM KCl is only partially retained when the other experimental conditions are the same as in Figure 1a. The adsorbed tubulin is released in two fractions, as in Figure 1a.

From these experiments, it is clear that, in the absence of guanine nucleotides, phosphocellulose-purified tubulin is adsorbed to phenyl-Sepharose. The amount of adsorbed protein

is influenced by the salt concentration.

Effect of Guanine Nucleotides on the Interaction of Tubulin with Phenyl-Sepharose. The influence of exogenous guanine nucleotides in the eluting buffer is most clearly demonstrated in a desorption process as shown in Figure 1c. While, in the absence of guanine nucleotides, no tubulin desorbs from the hydrophobic gel by elution with a 40 mM KCl–MM buffer (Figure 1a), the protein desorbs readily when 0.1–1 mM GTP or GDP is added to this buffer. After desorption of this tubulin fraction, the remaining tubulin cannot be eluted with water but is partially desorbed with a 50% (v/v) water–ethanol mixture. The addition of 0.5 mM GTP to an MM elution buffer containing 190 mM KCl also results in a gradual release of tubulin from the phenyl-Sepharose matrix.

In order to make sure that the elution of tubulin is not provoked by a specific interaction between guanine nucleotides and the phenyl-Sepharose gel, the passage of a GTP sample over the column was followed. GTP elutes without retention, in a symmetric peak without loss of product. Consequently, the desorption of tubulin on passage of GTP or GDP can only be explained by assuming that the binding of the guanine nucleotides to tubulin decreases its hydrophobicity.

Effect of Inorganic Phosphate and AlF_4^-. As the binding of inorganic phosphate and its structural analogue AlF_4^- increases the stability of microtubules (Carlier et al., 1988), we investigated whether these anions are able to modulate the hydrophobic behavior of tubulin by interfering with the effect of guanine nucleotides. Therefore, KCl present in eluting MM buffers of the previous chromatographic experiments is replaced by inorganic phosphate, sulfate, or AlF_4^- as follows. KCl (190 mM) is substituted respectively by a mixture of 160 mM KCl, 25 mM KH_2PO_4, and 5 mM K_2HPO_4, by 100 mM K_2SO_4, or by a mixture of 100 mM K_2SO_4, 1 mM $Al(NO_3)_3$, and 5 mM NaF. KCl (40 mM) is substituted respectively by a mixture of 25 mM KH_2PO_4 and 5 mM K_2HPO_4, by 15 mM K_2SO_4, or by a mixture of 15 mM K_2SO_4, 1 mM $Al(NO_3)_3$, and 5 mM NaF. The concentrations of Al^{3+} and F^- used are favorable for the formation of the AlF_4^- complex (Goldstein, 1964).

The presence of inorganic phosphate or its structural analogue AlF_4^- does not markedly influence the elution profile of tubulin on phenyl-Sepharose, but the addition of 0.5 mM GDP or GTP to the MM elution buffer containing the lower salt concentrations provokes an immediate elution of tubulin as in the absence of AlF_4^- (results not shown). These observations indicate that the property of guanine nucleotides to decrease the hydrophobicity of tubulin is not eliminated by inorganic phosphate nor by AlF_4^-.

Characterization of Eluted Tubulin Species. As tubulin partially elutes with water and partially with a more apolar solvent mixture, we investigated whether these two fractions contain α- and β-subunits. Electrophoresis on a 7.5% polyacrylamide gel containing 6 M urea shows that both subunits are present in both eluted fractions. The tubulin fraction eluted with water contains 52% α-subunits and 48% β-subunits ($\pm$4%). The tubulin fraction eluted with a 50% (v/v) ethanol–water mixture contains 54% α-subunits and 46% β-subunits ($\pm$4%).

Nucleotide Content of Tubulin Eluted with Water and Its Ability To Rebind GTP. For determination of the nucleotide content of the tubulin fraction eluted with water, the chromatography was scaled up. In a typical experiment (Figure 2), a 1.6 cm i.d. × 5 cm phenyl-Sepharose column is loaded with 20.2 mg of tubulin in 5 mL of MM buffer containing 100 mM K_2SO_4. The flow rate is 2 mL/min, and fractions of 1.50 mL are collected. In the fractions eluted with water, the tubulin content and the content of guanine nucleotides are analyzed by the methods described above. GTP is the only nucleotide eluting with the desorbed tubulin, and close to one GTP molecule is found per tubulin dimer.

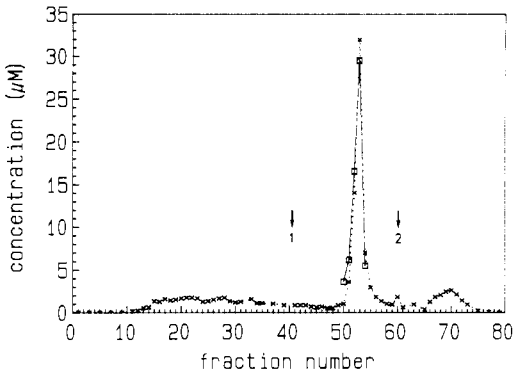

FIGURE 2: Comparison of tubulin and nucleotide content in the fraction released from a phenyl-Sepharose column by elution with water. Sample: 5 mL containing 20.2 mg of PC-tubulin. Column: 1.6 × 5 cm. Flow rate: 2 mL/min. Fractions: 1.5 mL. The elution buffer is MM buffer containing 100 mM K_2SO_4. It is changed to water at arrow 1 and to a mixture of 50% (v/v) ethanol–water at arrow 2. The tubulin content (×) and the nucleotide content (□) are determined as described under Materials and Methods. GTP is the only nucleotide found.

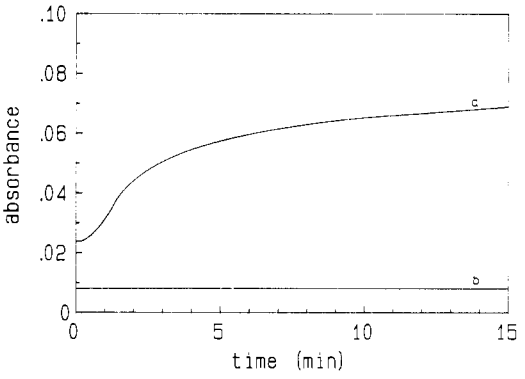

FIGURE 3: Assembly of tubulin, prepared as described under Results. Curve a: The polymerization buffer contains MM buffer with 15 mM K_2SO_4, 0.5 mM GTP, 2.3 M glycerol, 0.55 mg/mL tubulin, and 0.22 mg/mL MAPs (pH 6.5, at 37 °C). Curve b: Same conditions as in curve a, but without MAPs.

The ability of this tubulin to rebind GTP is checked by passing it through a centrifugation column after a 30-min equilibration period in MM buffer containing 15 mM K_2SO_4 and 0.2 mM GTP. In this experiment, the effluent contains 16.7 μM GTP and 8.7 μM tubulin, indicating that at least 90% of the tubulin eluted with water from phenyl-Sepharose does rebind the removed GTP.

Assembly Properties of Eluted Tubulin. The assembly ability of tubulin desorbed from the phenyl-Sepharose column is tested as follows. Tubulin is prepared by hydrophobic interaction chromatography in an experiment identical with that described in Figure 2. Immediately after elution with water, the tubulin fraction is mixed with a small volume of concentrated buffer to obtain MM buffer with 15 mM K_2SO_4 and tested for assembly. When a mixture of 0.53 mg/mL tubulin, 0.22 mg/mL MAPs, 2.3 M glycerol, 0.5 mM GTP, 15 mM K_2SO_4, and MM buffer is heated to 37 °C, the turbidity (absorbance at 350 nm) increases (Figure 3, curve a). During the following cooling the turbidity disappears (not shown). In the absence of MAPs, no increase of turbidity is observed at 37 °C (Figure 3, curve b). As the observed turbidity is clearly temperature- and MAPs-dependent, the results prove a good

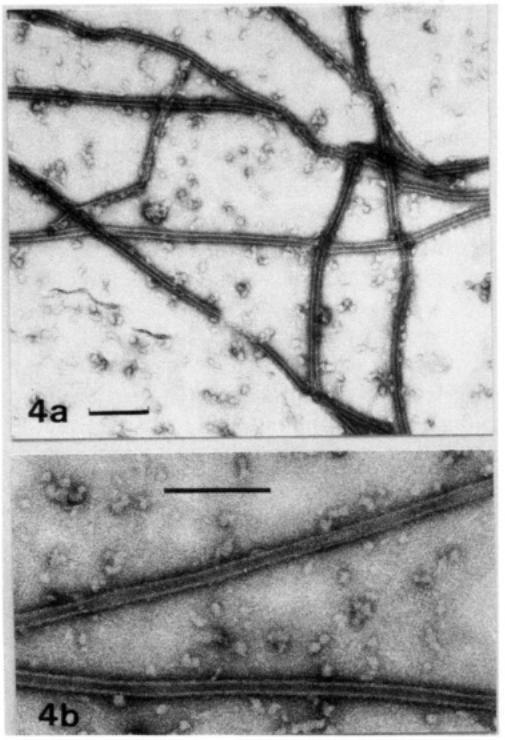

FIGURE 4: Electron micrographs showing the assembly of tubulin prepared as described in Figure 3, curve a. Note the negatively stained microtubules. In panel b, protofilaments of the microtubules may be distinguished. Magnification: (a) 40000×; (b) 70560×. Bar = 200 nm.

assembly and disassembly capacity of tubulin desorbed from a phenyl-Sepharose column with water as eluent.

An electron microscopic study of tubulin assembled in this way clearly reveals microtubules (Figure 4), in which protofilaments may be distinguished (Figure 4b).

Fluorescence of Mixtures of Tubulin and 1,8-ANS. 1,8-ANS and bis(1,8-ANS) are useful fluorescent probes for hydrophobic areas on protein molecules. Bis(1,8-ANS) binds strongly to tubulin and potently inhibits its polymerization into microtubules. The less strongly binding 1,8-ANS does not inhibit tubulin assembly (Horowitz et al., 1984). To reduce possible conformational changes induced by the probe, 1,8-ANS was chosen to estimate the influence of GTP on the exposure of hydrophobic areas in tubulin. Figure 5, curve b, shows that, at 12 °C, the fluorescence intensity of 1,8-ANS mixed with tubulin freshly prepared as in Figure 2 and in the presence of 0.2 mM GTP increases and quickly reaches a stable value which hardly varies in the next hours. In the absence of GTP, the same value of fluorescence intensity is also rapidly obtained, but during the next hours the fluorescence continues to increase. After 2 h, twice the value obtained in the presence of GTP is reached (Figure 5, curve a). These results indicate that, as well in the presence of GTP as in the absence of GTP, some hydrophobic sites quickly bind to 1,8-ANS. Moreover, in the absence of GTP, more 1,8-ANS slowly binds to hydrophobic sites during the next hours.

Two additional observations are worth mentioning. First, on addition of 0.2 mM GTP to a mixture of tubulin and 1,8-ANS (Figure 5, curve a, dashed lines), the fluorescence does not return to the value that is reached when GTP is added before 1,8-ANS (Figure 5, curve b). Second, when GTP and 1,8-ANS are added to tubulin incubated for 2.5 h (at 12 °C) after the elution from phenyl-Sepharose, about the same fluorescence intensity is quickly obtained (Figure 5, curve c) as with freshly chromatographed tubulin to which GTP and

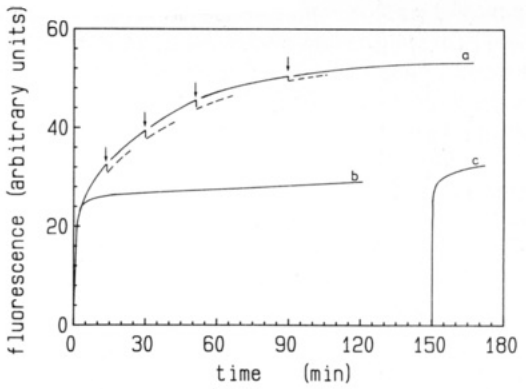

FIGURE 5: Strip chart tracing of the time course of fluorescence intensity for 1,8-ANS in the presence of tubulin with or without additional GTP at 12 °C. Excitation is at 350 nm and emission at 470 nm. Tubulin is freshly chromatographed from phenyl-Sepharose as in Figure 2. Curve a: The sample contains 0.125 mg/mL tubulin in MM buffer containing 15 mM K_2SO_4; at $t = 0$, 1,8-ANS is added to a concentration of 0.147 mM; in similar experiments (dashed lines added to curve a), GTP is added at different time points (arrows), to obtain a final concentration of 0.2 mM. Curve b: The sample is identical with that in curve a except it also contains 0.2 mM GTP; at $t = 0$, 1,8-ANS is added to a concentration of 0.147 mM. Curve c: The sample is identical with that in curve a; after an incubation of 150 min, GTP is added to a concentration of 0.2 mM, and 1,8-ANS is added to a concentration of 0.147 mM.

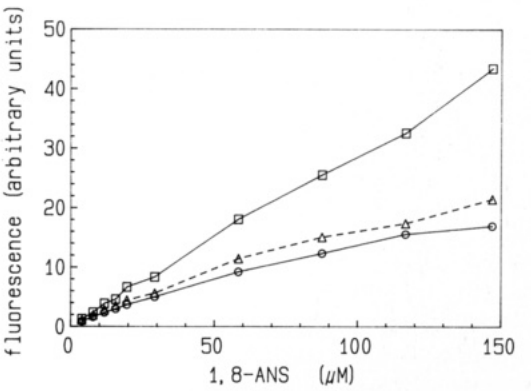

FIGURE 6: Fluorescence intensity of 1,8-ANS bound to tubulin as a function of 1,8-ANS concentration. Tubulin, at 1.2 μM in MM buffer containing 15 mM K_2SO_4, is mixed with 0–150 μM 1,8-ANS, and the fluorescence intensity is measured after a 10–15-min incubation period (△) and after 2 h (□). To tubulin is added GTP to 0.2 mM before the addition of 1,8-ANS, and the fluorescence intensity is measured after 1 h (○). The excitation wavelength is 350 nm and the emission wavelength 470 nm.

1,8-ANS are added (Figure 5, curve b). The first observation indicates that GTP does not remove the adsorbed 1,8-ANS from the hydrophobic areas of tubulin. This may suggest that the slowly increasing fluorescence and the accompanying 1,8-ANS adsorption result from a denaturation of the tubulin depleted of GTP at the exchangeable sites. However, if spontaneous denaturation of tubulin occurs in the 2.5-h incubation period of the experiment in Figure 5, curve c, we may expect a rapid 1,8-ANS adsorption and a rapid increase of the fluorescence to the same value as in the experiment shown in Figure 5, curve a. In Figure 5, curve c, however, the fluorescence does not quickly reach this high value, but rather the value as in Figure 5, curve b. Therefore, we accept that the increasing access of 1,8-ANS to hydrophobic regions of tubulin (Figure 5, curve a) does not result from a spontaneous tubulin denaturation, although a 1,8-ANS stimulated denaturation cannot be excluded.

The effect of different concentrations of 1,8-ANS added to tubulin at 12 °C and pH 6.5 is shown in Figure 6. In the

absence of exogenous nucleotides, the titration curve of nucleotide-depleted tubulin with 1,8-ANS, measured 10–15 min after mixing, approximately corresponds to the curve obtained in the presence of 0.2 mM GTP. After a 2-h incubation, the fluorescence intensities in the absence of GTP are increased at all 1,8-ANS concentrations.

The effect of AlF_4^- on the accessibility of 1,8-ANS to hydrophobic sites of tubulin was also investigated. As well in the absence as in the presence of GTP or GDP, the fluorescence intensities of 1,8-ANS-tubulin mixtures are not influenced by AlF_4^- at 12 and 30 °C (results not shown).

Discussion

The present study reveals a guanine nucleotide dependent hydrophobic behavior of tubulin. The strongest evidence comes from a GTP/GDP-stimulated release of tubulin from a phenyl-Sepharose gel. The tubulin fraction which conserves its ability to rebind nucleotides (Figure 1c) corresponds to the fraction eluted with water (Figure 1a). It contains one nucleotide molecule (GTP) per dimer, and the amount of α-subunits is approximately the same as the amount of β-subunits. This tubulin fraction is able to polymerize into microtubules. Its activity was determined in a quantitative way by measuring the amount of dimers rebinding GTP: at least 90% of the tubulin dimers rebind GTP. A direct correlation between the ability of tubulin to rebind nucleotides and its ability to polymerize has been demonstrated by Croom et al. (1986).

Our results indicate that not only is hydrophobic interaction chromatography useful for demonstrating the nucleotide-dependent hydrophobic character of tubulin, but it also appears to be a suitable method for obtaining active tubulin free of guanine nucleotides at the exchangeable sites. Other methods for removing the exchangeable nucleotides from tubulin, e.g., charcoal treatment (Penningroth & Kirschner, 1977) and gel filtration in Mg^{2+}-free buffer (Croom et al., 1985), deplete these sites only partially, and as much as 40% of the exchangeable nucleotides remains. Moreover, these methods for removing the exchangeable nucleotides result in an undetermined amount of inactive tubulin (Penningroth & Kirschner, 1978). The alkaline phosphatase treatment also results in an incomplete hydrolysis of the exchangeable nucleotides and a partial digestion of the nonexchangeable GTP (Bayley & Manser, 1985; Manser & Bayley, 1987). As active and inactive tubulin elute separately from the phenyl-Sepharose gel, a homogeneous active tubulin fraction, free of exchangeable nucleotides, is obtained by hydrophobic interaction chromatography.

On the basis of homologies with known nucleotide-binding proteins, Sternlicht et al. (1987) have assigned the sequences at which the guanine nucleotides bind to β-tubulin. The loops formed by residues 64–70 and 297–300 have a guanine-binding specificity. Residues 143–148 and 105–112 are suggested to bind the α- and β-phosphate groups of GDP, while the β- and γ-phosphates of GTP bind to residues 105–112 and 204–208. Using the hydrophobicity values proposed by Janin (1979), a graph of the hydrophobicity of the amino acid residues as a function of the residue number indicates that the sequence 141–154 of the β-subunit of porcine tubulin represents a strikingly large hydrophobic region. As this sequence contains a phosphoryl-binding loop (Sternlicht et al., 1987), it may be responsible for the enhanced hydrophobic interaction of tubulin depleted of exchangeable guanine nucleotides.

The information obtained by chromatography on phenyl-Sepharose and that obtained by the 1,8-ANS fluorescence experiments are complementary. As observed by other authors (Bhattacharyya & Wolff, 1975; Steiner, 1980; Horowitz et al., 1984), the fluorescence measurements on 1,8-ANS indicate that even GTP-saturated tubulin contains easily accessible hydrophobic sites. Apparently, these sites are not large and/or numerous enough to retain the protein on phenyl-Sepharose. The similarity of the titration curve of GTP-saturated tubulin and the curve of nucleotide-depleted tubulin, obtained immediately after 1,8-ANS addition (Figure 6), indicates that the access to this primary hydrophobic site(s) is nucleotide-independent. The time-dependent increase of the 1,8-ANS fluorescence, which is only observed in the absence of GTP (Figure 5, curve a), may be related to a slow 1,8-ANS binding to the hydrophobic sites shielded in the presence of nucleotides. The slow penetration of 1,8-ANS into these sites contrasts with the rapid binding of tubulin to phenyl-Sepharose. However, the latter interaction is stimulated by the high density of hydrophobic sites on the gel and by the higher salt concentration used on application of tubulin.

The main difference between the tubulin adsorption to 1,8-ANS and the adsorption to phenyl-Sepharose is the reversibility of the latter process: addition of 0.1 mM GTP or GDP to 40 mM KCl (or 15 mM K_2SO_4)–MM elution buffer results in an immediate desorption of tubulin from phenyl-Sepharose (Figure 1c), while the addition of GTP to a mixture of 1,8-ANS and nucleotide-depleted tubulin (Figure 5, curve a, dashed lines) does not cause a drop of the fluorescence to the value reached when GTP is added to tubulin before 1,8-ANS is added (Figure 5, curve b).

The interaction of tubulin with the macromolecular probe phenyl-Sepharose is in a reversible way determined by GTP and GDP, indicating that the binding of these nucleotides at the exchangeable binding sites of the tubulin dimers screens—in a direct or indirect way—hydrophobic sites from the external medium. Moreover, the increase of the enthalpy and entropy, and the negative heat capacity change accompanying the formation of microtubules (Lee & Timasheff, 1977; Hinz & Timasheff, 1986), as well as other properties mentioned in the introduction, suggests an important involvement of hydrophobic interaction in the formation of microtubules. It has also been demonstrated (Carlier et al., 1988) that GTP hydrolysis and the subsequent release of the inorganic phosphate regulate respectively stabilization and destabilization of microtubules. To correlate all these data, we hypothesize that, on hydrolysis of GTP, hydrophobic sites are easily exposed, stabilizing or strengthening the intramolecular interactions in microtubules. Reshielding the hydrophobic regions by GDP should require the release of the phosphate groups which are cleaved from GTP but not immediately desorbed from tubulin. This should occur at the depolymerization end of microtubules. Figure 7 represents a scheme of this possible exposing and reshielding of a hydrophobic area.

To test our hypothesis, we studied the influence of inorganic phosphate and its analogue AlF_4^- on the stabilization of the hydrophobic interactions in the presence of guanine nucleotides. In our experiments, the nucleotide (GTP or GDP) stimulated release of tubulin from phenyl-Sepharose is not reduced in the presence of inorganic phosphate or AlF_4^-. Besides, the fluorescence intensity does not reach higher values when the tubulin solution contains AlF_4^- on the addition of guanine nucleotides and of 1,8-ANS. These results indicate that exogenous inorganic phosphate and its analogues do not stabilize in an important way the exposed hydrophobic regions of nonpolymerized tubulin in the presence of guanine nucleotides. These results do not confirm the proposed hypothesis

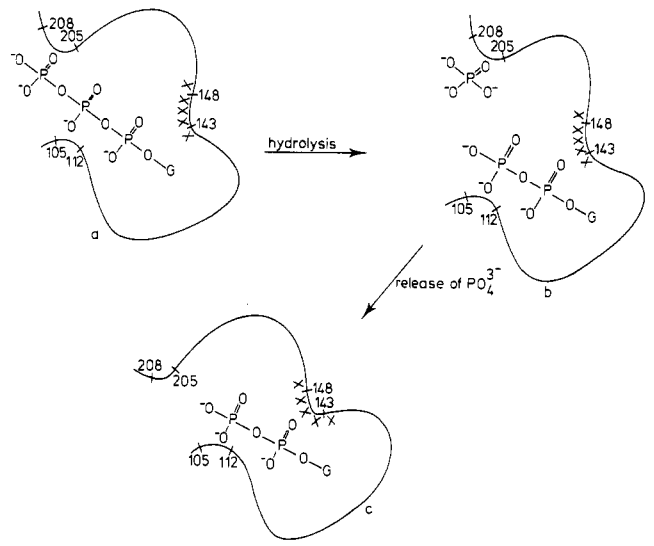

FIGURE 7: Schematic representation of GTP hydrolysis, release of the cleaved phosphate, and the related exposure and reshielding of a hydrophobic area in a β-tubulin segment incorporated in a microtubule. XXX represents a hydrophobic sequence, and G represents a guanosine group. In this scheme, the positions of the nucleotide-binding sites assigned by Sternlicht et al. (1987) are used. (a) the β- and γ-phosphates of GTP bind to β-tubulin loops 105–112 and 205–208, while the phosphoryl-binding region 143–148 is hindered to bind the α-phosphate group. (b) Steric hindrance and electrostatic repulsion of the cleaved phosphate may improve the exposure of some hydrophobic sites, which participate in stabilizing intramolecular interactions. (c) Following the release of the inorganic phosphate, a binding of the loop 143–148 to the α-phosphate of GDP is allowed, reshielding in this way the hydrophobic area(s) involved in the intramolecular interactions.

but are in agreement with the recent findings of Carlier et al. (1989), indicating that exogenous inorganic phosphate and its structural analogues bind with high affinity to tubulin incorporated in microtubules but do not bind to dimeric or oligomeric tubulin. This means that the influence of exogenous phosphate and its analogues on the ability of guanine nucleotides to shield the hydrophobic sites cannot be studied in a direct way.

In conclusion, this paper describes a new method for removing the exchangeable nucleotides from tubulin. At least 90% of this tubulin rebinds GTP, and microtubules are formed on polymerization. Our results indicate that guanine nucleotides can regulate in a reversible way the exposure of hydrophobic groups on tubulin. The thermodynamic properties accompanying the microtubule assembly, as well as the regulatory role of guanine nucleotides in polymerization and depolymerization, allow us to speculate that a control of this hydrophobic exposure by guanine nucleotides is important for the stability of microtubules.

ACKNOWLEDGMENTS

We thank Prof. Dr. M. Joniau for helpful discussions, W. Noppe for his expert technical assistance in the high-performance liquid chromatography, and B. Strynck for excellent technical assistance in the electron microscopy and photography.

Registry No. GTP, 86-01-1; GDP, 146-91-8.

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