MICROTUBULAR PROTEIN REACTION WITH NUCLEOTIDES

Michael Jacobs† and Michael Caplow††

†Medical Research Council Cell Biophysics Unit, King's College, 26-29 Drury Lane, London WC 2, England and ††Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514

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The dissociation constants for GTP and GDP with tubulin were determined to be equal to 1.1 ± 0.4 x 10^{-7} M and 1.5 ± .6 x 10^{-7} M (4°), respectively. A lower limit for the dissociation constant for ATP was established as equal to 6 x 10^{-4} M. The equivalent binding of GTP and GDP is not readily consistent with a mechanism in which the role of GTP in microtubule assembly is to bind to the protein to induce a conformation which is able to polymerize. An ATP-induced polymerization of tubulin apparently involves a transphosphorylation reaction in which GTP is formed and mediates the assembly. For this reaction to occur with desalted tubulin trace amounts of GDP are required; in the reaction of 0.1 mM ATP with 22.0 μM tubulin, 0.1 μM GDP induces about 80% as much tubule formation as is seen with 0.1 mM GTP alone.

The tubulin dimer has two guanine nucleotide binding sites, a rapidly dissociating (E) site and a nondissociating (N) site (1). The E site appears to bind guanine nucleotides specifically and an N-site bound GDP may be converted to GTP by a transphosphorylation reaction (1,2). Occupancy of the E-site by GTP is necessary for rapid assembly of tubulin into microtubules and the polymerization is accompanied by hydrolysis of the bound nucleotide[1]. Although no significant binding of ATP to the E-site has been observed (1,2) it was reported that this nucleotide will support polymerization (4,5). However, it was noted (2) that this result could be accounted for by a pathway involving a transphosphorylation reaction in which GTP is synthesized from ATP and then utilized in the polymerization process.

We report here on the nucleotide binding to tubulin and the nucleotide concentration dependence for microtubule assembly.

Methods

Tubulin, which was prepared from pig brain by a published procedure (6), was purified by an additional polymerization-depolymerization cycle immediately before use. The protein used in kinetic studies was desalted to remove loosely

1. There is disagreement as to whether the N-site or E-site nucleotide is cleaved; cf. ref. 1 and 3.

bound and free nucleotides by passage through a Sephadex G-25 column (0.9 x 25 cm) which had been equilibrated with reassembly buffer. This was found to be unnecessary for protein used in the equilibrium binding studies. Rates of polymerization were determined from spectrophotometric measurements (4) at room temperature (22° ± 2) and in all cases it was shown that the reaction could be fully reversed by 2.5 mM calcium. In a number of experiments it was shown by electron microscopy that native-looking microtubules formed in the reaction mixtures used in the kinetic measurements.

Results

Nucleotide Binding: The Hummel-Dryer column equilibrium method (7) was used to determine the equilibrium constant for GTP binding to the E-site. A competition method, which permits the use of very high ligand concentrations was used to evaluate GDP and ATP binding. The column elution pattern observed in a representative experiment is given in Figure 1. The fact that the eluate radioactivity following the tubulin-GTP peak returns to the baseline level indicates that the column passage time is sufficient to permit equilibrium to be established. This was further verified by the constancy of the size of the tubulin-GTP peak when the column length was halved, to 25 cm. The method permits determination of the equilibrium constant without removal of nucleotides contaminating the protein since these are displaced from the protein during the passage through the column. The results from 15 column runs are presented in the form of a reciprocal plot in Figure 2 and the number of GTP binding sites/tubulin dimer, determined from the intercept, is 0.53. This low value may be caused by scatter in the experimental results, which was especially marked because of the necessity for using a number of different protein preparations for the measurements on different days. Also, it is influenced by the presence of non-tubulin protein contaminants and nonbinding forms of tubulin. The dissociation constant, which is not influenced by the presence of inactive protein, is calculated to be $1.1 \pm .4 \times 10^{-7}$. The dissociation constant for GDP was determined with 1.5×10^{-7} M GTP and 0.015-1.5×10^{-7} M GDP competing with GTP binding. This was reduced by 19-96% at different GDP concentrations and the calculated dissociation constant from these measurements, assuming that GDP binding is competitive with that for GTP[2], was in

2. The binding of GDP appears to be competitive with GTP, but there is insufficient data to conclude this with certainty.

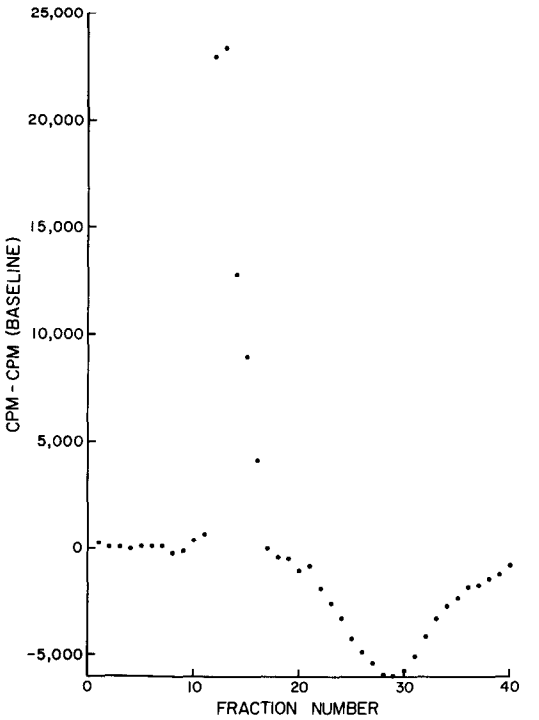

Fig. 1 Gel filtration of tubulin in Sephadex G-25 containing 7.6×10^{-8} M GTP. Tubulin (2.95 μM) was reacted with 7.6×10^{-8} M GTP in 100 mM MES, pH 6.6, with 0.5 mM Mg and 1 mM EGTA (reassembly buffer). One ml was then passed through a 0.5 x 50 cm column which had been equilibrated with [^{32}P] GTP. Radioactivity in the weighed column fractions was measured.

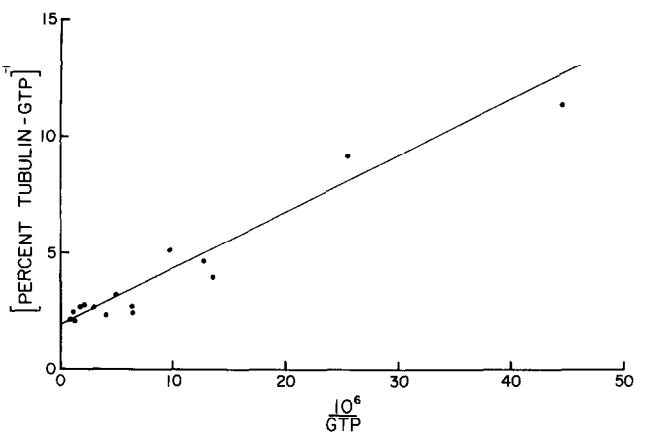

Fig. 2 Dependence of (fraction of tubulin complexed with GTP)$^{-1}$ on (GTP)$^{-1}$ at 4°.

the range $1.5 \pm .6 \times 10^{-7}$ M. The protein, therefore, has about equal affinity for GTP and GDP. The K_D for GDP was found to be the same at room temperature (22° ± 2) and at 4°. Binding of 7.6×10^{-8} M GTP is not measurably influenced

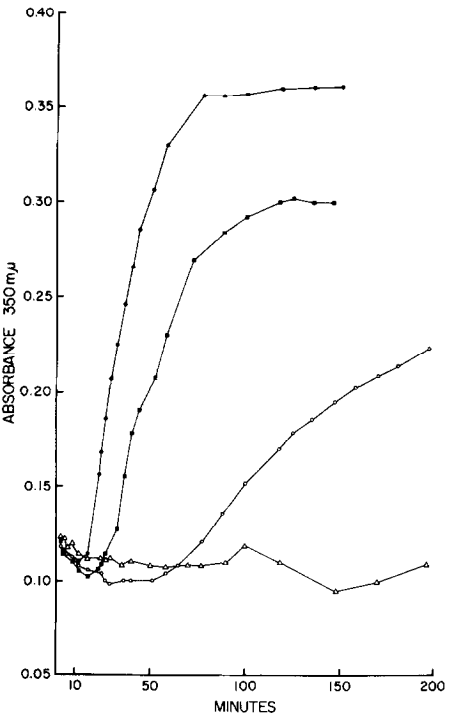

Fig. 3 Rate of microtubule formation with 0.11 mM ATP at various GDP concentrations. Reactions were run at room temperature (22 ± 2°) with 20 μM tubulin in reassembly buffer. The curves with progressively increasing slopes were measured with 0, 1.7, 4.27 and 210 μM GDP, respectively.

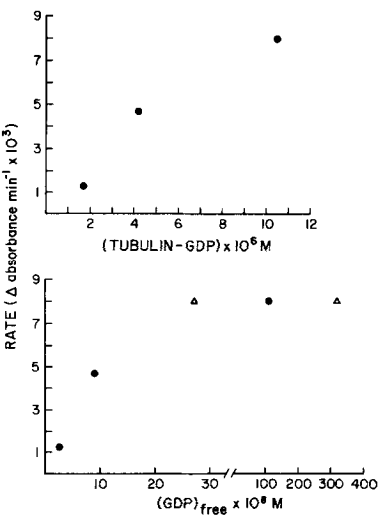

Fig. 4 Dependence of the rate of tubulin polymerization on the tubulin-GDP concentration (upper graph) and the unbound GDP concentration (lower graph). The concentration of the two GDP species were calculated from the total GDP concentration and the equilibrium constant for nucleotide binding. The results obtained in the experiment described in Fig. 3 (●) as well as the normalized results obtained in an additional experiment (△) are presented.

by ATP at concentrations to 5.3×10^{-4} M. Assuming that a 33% decrease in GTP bound would go undetected, a lower limit for the dissociation constant for ATP equal to 0.6 mM may be calculated. A recent study of nucleotide binding to tubulin using a nitrocellulose filter assay method gave dissociation constants equal to 0.5 and 1.9 μM for GTP and GDP, respectively (8). Very weak ATP binding was observed (8). The Hummel-Dryer method has previously been used to measure GTP binding to tubulin at 2° (9) and a dissociation constant equal to 0.4 μM was estimated. This value may only be approximately correct since virtually no variation in the amount of GTP bound was seen over the very limited (0.5-2.0 μM) GTP concentration range used.

Polymerization Kinetics: With nondesalted tubulin it was found that ATP (0.1 mM) induced polymer formation. However, on removal of free and exchangeable nucleotides by gel filtration there is no polymerization with ATP without added GDP (Figure 3). Results equivalent to those shown in Figure 3 were obtained with a number of batches of tubulin. We have, however, found that with some protein preparations the length of the lag preceding polymerization is considerably shortened and the rate is appreciably greater than that shown here. With such protein preparations the rate of the GTP-induced polymerization is not abnormally rapid. This variability in the rate of the ATP-induced polymerization presumably reflects variations in the concentration of the transphosphorylase (see below) in the tubulin preparations. It is seen in the results given in Figure 3 that there is a time lag preceding polymerization, the length of which is inversely proportional to the GDP concentration. The dependence of the rate of polymerization on the GDP concentration (Figure 4) shows a saturation effect, becoming independent of concentration at high GDP concentrations. The rate at these saturating concentrations is less (about 3-fold) than that seen when the polymerization of the same protein preparation is induced with GTP. GDP apparently acts catalytically in the polymerization, as indicated by the fact that the amount of nucleotide necessary to induce polymerization is considerably less than the tubulin concentration. For example, with 0.1 μM GDP and 0.1 mM ATP the final absorbance

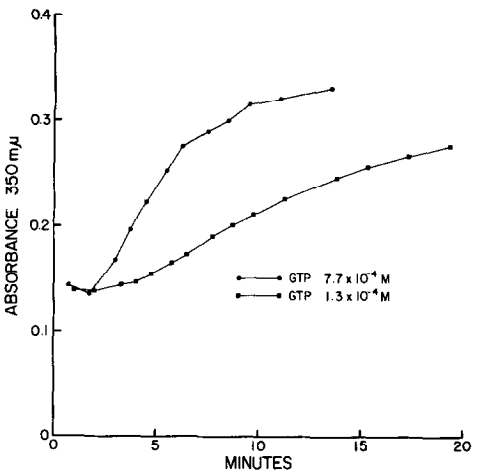

Fig. 5 GTP-dependent polymerization measured with 17 μM tubulin in reassembly buffer.

seen after polymerization of 22.0 μM tubulin was found to be about 80 percent of that when the polymerization was induced with 0.1 mM GTP. It was previously demonstrated (4) that the absorbance measured at 350 nm provides a correct estimate of the amount of high molecular weight product formed. GDP is a potent inhibitor for the GTP-induced polymerization: no tubules were formed in a mixture containing 0.113 mM GTP and 0.27 mM GDP. This inhibition is consistent with the comparable GDP and GTP affinity described above. Using both nondesalted and desalted tubulin it was found that 1.2 mM GDP inhibits the 0.1 mM ATP-induced polymerization. The rate of polymerization at two GTP concentrations is shown in Figure 5.

Discussion

The GDP requirement for ATP-induced microtubule assembly is consistent with a reaction sequence (eq. 1) in which GTP is formed

(1) GDP + ATP $\xrightarrow{\text{step 1}}$ ADP + GTP + tubulin $\xrightarrow{\text{step 2}}$ tubules + GDP

by a transphosphorylation reaction (step 1), with the so-formed nucleotide mediating the polymerization (step 2). In the earlier report that the ATP-dependent reaction does not require GDP (4) it was not established that the protein was

fully depleted of nucleotide; only very low levels of GDP are required in the ATP-induced reaction.

It was previously demonstrated that the tubulin-associated transphosphorylase catalyzes GTP formation at the N-site (1.2). The need for GDP in the ATP-induced assembly reaction may reflect a requirement for nucleotide at the E-site for the transphosphorylation and/or a requirement for GDP or GTP at the E-site for polymerization to occur. It is not known whether the transphosphorylase catalyzes GTP formation with the added GDP. The fact that the GDP concentration needed for polymerization is below that of the protein concentration is consistent with a mechanism in which only partial occupancy of the E-site is required for polymerization to occur. A mechanism in which the E-site nucleotide is released after polymerization and further utilized is unlikely since it was shown that the E-site nucleotide is retained on tubule formation (1).

The rate of the ATP-induced polymerization becomes independent of the GDP concentration at high concentrations (Figure 4). This saturation effect is consistent with at least two mechanisms. In one, the saturation reflects a change in rate-limiting step, from rate-limiting transphosphorylation (step 1 in eq. 1) at low GDP concentrations, to rate-limiting microtubule assembly (step 2) at high GDP concentrations. An equally likely mechanism involves rate-limiting transphosphorylation at all GDP concentrations. In this case the invariance of the rate at high GDP concentrations reflects the saturation of the transphosphorylase with GDP. For this mechanism the substrate for the transphosphorylase can be either free GDP or a tubulin-GDP complex; the latter possibility is favored from quantitative analysis of the results. From the curves given in Figure 4 it can be calculated that if free GDP is the substrate the apparent K_D is equal to approximately 6×10^{-8} M. This value is very much lower than the K_m for nucleotide diphosphates seen with a variety of transphosphorylases (10), for which the K_m is in the range 0.05-1 mM; it therefore appears that this is not the correct route for GTP synthesis. For a reaction in which the phosphate

acceptor is a tubulin-GDP complex a very reasonable value for K_m equal to approximately 3.5 μM may be calculated (Figure 4). Proof of the existence of a tubulin binding site on the transphosphorylase would be significant since it would implicate this catalyst in the physiological microtubule assembly process.

In the results described in Figure 5 it can be seen that the rate of polymerization is dependent on the GTP concentration under conditions where it can be calculated that the E-site is fully saturated with GTP; an equivalent result is described elsewhere (11). This can be taken to indicate that GTP interacts with some site other than or in addition to the E-site in the assembly reaction.

The equivalent binding seen for GDP and GTP is important. It indicates that the nucleotide influence on polymerization is unlikely to occur by an allosteric mechanism in which GTP serves to shift an equilibrium in which the protein exists in polymerizable and nonpolymerizable conformations, with the latter species predominating in the absence of GTP. GDP does not induce polymerization and if GTP but not GDP binding is coupled with a thermodynamically unfavorable protein conformation change it would be expected that GDP binding would be very much tighter than that of GTP; this is not observed. The alternate possibility where there is a fortuitous cancellation of effects such that the higher affinity of GTP is almost exactly balanced by the requirement for a thermodynamically unfavorable protein conformation change associated with its binding is not ruled out, but is considered unlikely.

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