In Vitro Assembly of Microtubule Protein with GTP and 2′dGTP: Kinetic Evidence for a Preassembly Conformational Change†

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ABSTRACT: The assembly of chick brain microtubule protein in a NaCl-supplemented buffer has been examined with respect to nucleation and the subsequent elongation as a function of the nucleotide (GTP *vs* 2′dGTP), and the protein and nucleotide concentrations. The kinetics suggest that unassembled tubulin can exist in two conformational states (termed $Tu^{1,GTP}$ and $Tu^{2,GTP}$ when GTP is bound to the exchangeable site), with $Tu^{1,GTP}$ contributing to nucleation and $Tu^{2,GTP}$ participating in elongation. The extent of self-nucleation is proposed to be determined, in part, by the rate constant governing this conformational change. This analysis contrasts with that of earlier studies, which concluded that the number of subunits interacting to form an effective nucleus could be estimated from the dependency of self-nucleation on the initial concentration of unassembled tubulin.

An analysis of microtubule assembly *in vitro* has proved instructional to an understanding of the behavior of microtubules *in vivo*. The *in vitro* assembly involves an initial nucleation process followed by elongation toward steady state. By contrast, it is increasingly evident that microtubules are nucleated *in vivo* by microtubule organizing centers, and that this may involve γ-tubulin since it is specifically localized at such nucleation sites (e.g., Oakley et al., 1990; Horio et al., 1991). While the kinetically unfavorable nucleation observed *in vitro* may therefore not apply *in vivo*, an analysis of this process may highlight a property of tubulin which contributes to its cellular function.

Nucleation *in vitro* has been assumed to involve the interaction of a specific number of tubulin dimers (Carlier & Pantaloni, 1978; Engelborghs et al., 1980; Robinson & Engelborghs, 1982), such that

$$[M] = K_n[Tu^{GTP}]^{n/2}$$

$$\log [M] = \log K_n + n/2 \log [Tu^{GTP}]$$

Widely varying values have, however, been reported. Analysis of the initial rate of microtubule assembly, measured turbidometrically, as a function of the initial tubulin concentration indicated a two-step interaction of seven tubulin dimers (Voter & Erickson, 1984). Determination of the number of nucleation events from the subsequent elongation kinetics indicated that interactions between two components, such as two tubulin dimers or a dimer and a microtubule-associated protein, were required for the nucleation of unfractionated microtubule protein (Engelborghs et al., 1977). A similar number was obtained for the nucleation of pure tubulin assembled with dimethyl sulfoxide (Robinson & Engelborghs, 1982), clearly demonstrating that nucleation does not require a microtubule-associated protein. Higher numbers have been reported for the nucleation of pure tubulin assembled with glycerol and Mg^{2+} (10−12; Carlier & Pantaloni, 1978) and with 1.4 M dimethyl sulfoxide (4−5; Algaier & Himes, 1988).

The elongation of microtubules prior to the onset of dynamic instability is described by

$$-d[Tu^{GTP}]/dt = k_{+1,GTP}[Tu^{GTP}][M] - k_{-1,GTP}[M]$$

The pseudo-first-order rate constant ($k_{+1,GTP}[M]$)[1] would therefore be predicted to be directly proportional to the number of nucleation events provided there is neither end-to-end annealing nor dynamic instability, both of which would reduce [M]. These conditions are difficult to achieve when analyzing the assembly of pure tubulin. By contrast, the assembly of a stoichiometric mixture of MAP2 and tubulin dimers, using a buffer which dissociates the MAP2−tubulin oligomers (Burns, 1991a), satisfies both conditions. For example, there is little or no end-to-end annealing for this protein with this buffer (Idriss et al., 1991). Similarly, little or no dynamic instability is detected until shortly before the onset of steady state. This is demonstrated by the linearity of the pseudo-first-order plot from the end of nucleation until immediately before steady state (Burns, 1991a). It is also demonstrated by the 1:1 stoichiometry of GTP hydrolysis to subunit addition during the elongation phase (Burns, 1991b) since dynamic instability involves the loss of Tu^{GDP} subunits from the microtubule end. The precise values for $k_{+1,GTP}$ and $k_{-1,GTP}$ have been determined for the

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[1] Abbreviations: $[Tu^{GTP}]$, $[Tu^{2'dGTP}]$, or $[Tu^{GDP}]$, concentration of tubulin subunits with GTP, 2′dGTP, or GDP bound to the exchangeable sites; [M], molar concentration of microtubule ends; $k_{+1,GTP}$, $k_{+1,2'dGTP}$, $k_{-1,GTP}$, and $k_{-1,2'dGTP}$, association and dissociation rate constants governing the addition and loss of Tu^{GTP} or $Tu^{2'dGTP}$ subunits to the microtubule end; K_n, nucleation association constant; k_N, nucleation rate constant; n, apparent number of tubulin dimers required for nucleation; $Tu^{1,GTP}$ and $Tu^{1,2'dGTP}$ and $Tu^{2,GTP}$ and $Tu^{2,2'dGTP}$, two postulated conformational states of unassembled Tu^{GTP} and $Tu^{2'dGTP}$ tubulin dimers (see Discussion); k_c, rate constant(s) governing this conformational change.

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assembly of chick brain MAP2−tubulin under these buffer conditions (Burns, 1991b; Symmons & Burns, 1991), and this permits the extent of nucleation to be calculated from the subsequently observed elongation kinetics. By contrast, pure tubulin microtubules exhibit both end-to-end annealing and extensive dynamic instability, both of which adversely affect the ability to determine the number of nucleation events from the elongation kinetics and from the final microtubule lengths.

We have also exploited the finding that various modifications of GTP, notably 2′dGTP and 2′3′ddGTP, are more efficient than GTP at nucleating microtubule assembly (Hamel et al., 1984). Analysis of the nucleation and subsequent elongation with GTP and 2′dGTP suggests that unassembled Tu^{GTP} subunits exist in two differing conformational states, and that one ($Tu^{1,GTP}$ and its 2′dGTP equivalent) is selectively used during nucleation while the other ($Tu^{2,GTP}$ and its 2′dGTP equivalent) is required for elongation.

MATERIALS AND METHODS

Microtubule protein, consisting of a stoichiometric MAP2−tubulin mixture (Burns & Islam, 1984a), was purified through two cycles of assembly from day-old chick brains (Islam & Burns, 1985) in a buffer consisting of 100 mM Mes, 2.5 mM EGTA, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, and 0.1 mM EDTA (pH 6.4 with KOH) and then stored under liquid nitrogen. Immediately before use, the protein was depleted of exogenous nucleotides by elution through a 0.9 × 28 cm Sephadex G-50 column equilibrated with the above buffer supplemented with 67 mM NaCl (Burns, 1991a). Elution under these conditions reduces the occupancy of the β-tubulin exchangeable site to 0.25 mol of GDP/mol (unpublished observation). This residual GDP was fully exchangeable with exogenously added nucleotide (e.g., [^{3}H]GTP, [^{3}H]GDP, or [^{3}H]2′dGTP); *i.e.*, the residual GDP did not indicate that ~25% of the protein had abnormal nucleotide binding properties. All experiments used the NaCl-supplemented assembly buffer in order to dissociate the MAP2−tubulin oligomers and so reduce the assembly kinetics to an approximately pseudo-first-order reaction from nucleation until shortly before steady state (Burns, 1991a; Symmons & Burns, 1991).

The eluted protein was immediately adjusted to 100 μM GTP or an appropriate concentration of 2′dGTP, to minimize any loss in assembly-competency resulting from depleting the exogenous nucleotide. The samples were degassed and transferred to spectrophotometer cells prewarmed to 25 °C. They were then inserted into the Peltier-controlled thermostatic block of the Beckman DU-8 spectrophotometer, which had been prewarmed to 37 °C. This protocol minimized the warming time (to ≥ 60 s, compared with a typical assembly time of 10 min), while eliminating errors in the initial measurement at A_{350} resulting from the condensation caused by transferring the cold assay sample into a cuvette prewarmed to 37 °C. An NTP-regenerating system (consisting of 1 mM phosphoenolpyruvate and 60 $\mu g \cdot mL^{-1}$ pyruvate kinase) was only included when stated. The assembly was monitored turbidometrically at 350 nm and expressed as micromolar tubulin assembled by assuming a scattering coefficient of 44 μM tubulin (*i.e.*, the assembly of 44 μM tubulin dimers into microtubules increases the absorption at A_{350} by 1.0; Burns, 1991a). Values of $k_{+1,GTP}$[M] or $k_{+1,2'dGTP}$[M] were calculated from the pseudo-first-order plots, *i.e.*, the instantaneous rate of assembly *vs* the concentration of tubulin yet to be assembled (Burns, 1991a). In each experiment, the assembly-competency was determined from the slope of the steady-state extent of assembly, with 100 μM GTP and the GTP-regenerating system, as a function of the initial tubulin concentration. Alternatively, the sum of the extent of assembly with GTP and the elongation critical concentration [*i.e.*, $k_{-1,GTP}/k_{+1,GTP}$; see Burns (1991b)] was expressed as a function of the total tubulin concentration. In all cases, the assembly-competency was 75 ± 5%.

Microtubule lengths were determined by fixing aliquots with 0.4% glutaraldehyde in assembly buffer and then immunostaining (Symmons & Burns, 1991), or by fixing with 0.1% glutaraldehyde and negatively staining with 1% uranyl acetate. Protein concentrations were determined (Hartree, 1972) using bovine serum albumin as a standard, and calculating the tubulin concentration by assuming that it comprises 80% of the total microtubule protein (Burns & Islam, 1984a). Microtubule number concentrations were calculated from the assembled tubulin concentrations and the measured microtubule lengths and by assuming 1750 dimers$\cdot\mu m^{-1}$ (*i.e.*, a 14-protofilament microtubule). Nucleotide concentrations were determined from the absorbancy at A_{260}.

All reagents were purchased from Sigma Chemical Co., Ltd., with the exception of the primary and secondary antibodies (Serotec, Kidderminster, Oxon, U.K.).

RESULTS

Increasing concentrations of nucleotide-depleted microtubule protein were assembled to immediately before steady state with 100 μM GTP and the GTP-regenerating system, at which time samples were removed to determine the mean microtubule lengths by immunofluorescence. The mean lengths decrease with the increasing initial tubulin concentrations due to the more efficient nucleation; the method potentially underestimates [M] at higher tubulin concentrations due to the intrinsic difficulty in accurately measuring the lengths of very short microtubules. The mean lengths were used, together with the observed extents of assembly, to calculate [M] for each protein concentration, corrected for the assembly-competency of the protein. log [M] is directly proportional to log [Tu^{GTP}] (Figure 1); the slope is 2.25, indicating that an effective nucleus requires the *apparent* interaction of four to five tubulin dimers (see Discussion).

These data, when presented in the form of $k_{+1,GTP}$[M] *vs* [M] (Figure 2), confirm that the pseudo-first-order rate constant is directly proportional to [M] for the assembly at low initial protein concentrations, and hence inefficient nucleation. The data deviate from this prediction at high initial protein concentrations, *i.e.*, when there is efficient nucleation, such that $k_{+1,GTP}$[M] is always less than 12 × 10^{-3} s^{-1} (Figure 2). This deviation is not a consequence of underestimating the extent of assembly since the turbidometric values corresponded to the amount of pelleted protein (100000*g*, 5 min; data not shown). Nonlinear plots of $k_{+1,GTP}$[M] *vs* [M] were also obtained if [M] was varied by adding exogenous seeds (data not shown). Furthermore, the

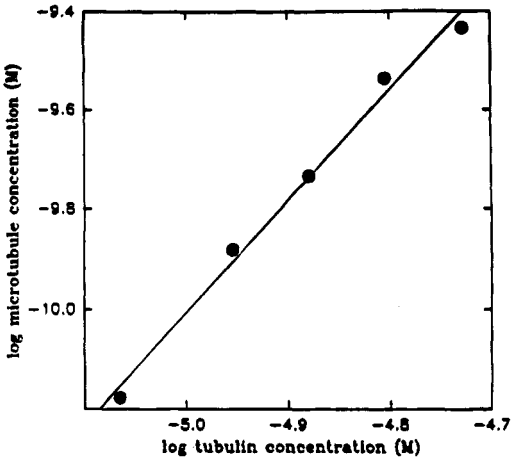

FIGURE 1: log/log plot of microtubule nucleation as a function of the initial concentration of assembly-competent tubulin. Increasing concentrations of nucleotide-depeleted microtubule protein were assembled to steady state with 100 μM GTP and a GTP-regenerating system. Nucleation [M] was calculated from the extent of assembly at a time immediately before steady state, and the mean lengths of the microtubules were determined by immunofluorescence. The assembly-competency of the protein was determined from the extent of assembly at steady state as a function of the initial protein concentration, corrected for the effects of dynamic instability (Burns, 1991a).

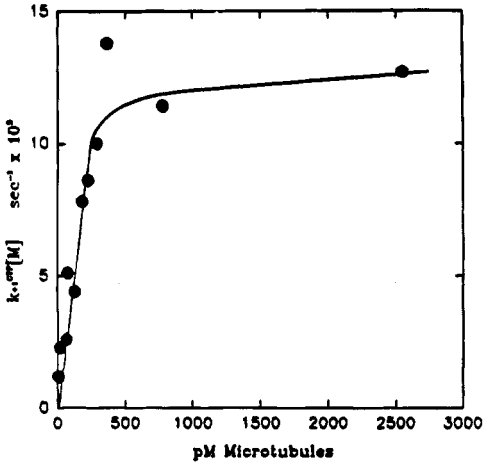

FIGURE 2: Values of $k_{+1,GTP}$[M] as a function of the concentration of nucleated microtubules [M]. Nucleotide-depleted microtubule protein was assembled with 100 μM GTP and a GTP-regenerating system. Values of $k_{+1,GTP}$[M] were derived from the instantaneous rate of assembly and the concentration of tubulin yet to be assembled [see Figure 3 and Burns (1991a)], and [M] as described in Figure 1.

nonlinearity is not due to the direct addition of MAP2–tubulin oligomers, since (i) such oligomers are dissociated by the NaCl-supplemented assembly buffer (Burns, 1991a), (ii) *higher* than predicted values of $k_{+1,GTP}$[M] are observed when oligomers are directly incorporated (Burns & Islam, 1984b; Bayley et al., 1985; Islam & Burns, 1986; Barton et al., 1987), and (iii) the pseudo-first-order plots are linear (Figure 3, top and bottom panels) yet are biphasic when both tubulin dimers and MAP2–tubulin oligomers can be added to the elongating microtubule (Burns & Islam, 1984b; Islam & Burns, 1986). The linearity of these plots also eliminates the possibility that the lower than predicted values of $k_{+1,GTP}$[M] at high initial protein concentrations are due to a concentration-dependent "poisoning" of some of the ends.

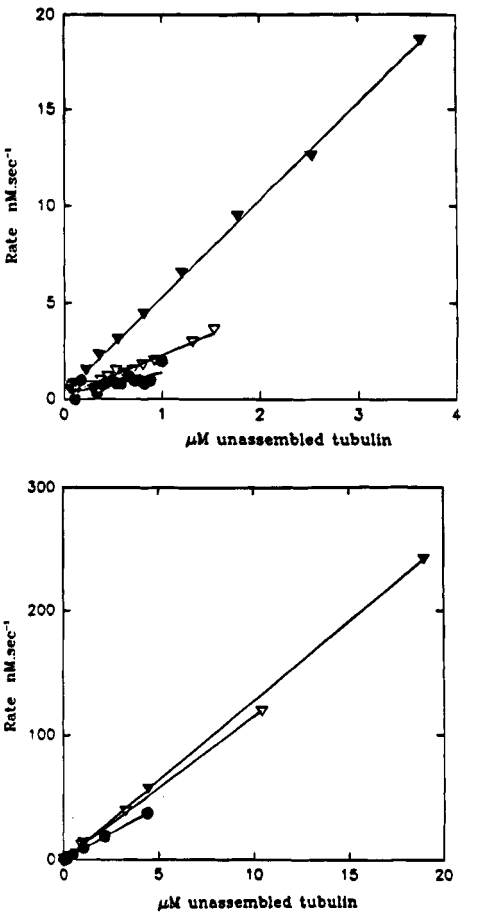

FIGURE 3: Pseudo-first-order plots (Burns, 1991a) for the assembly of (A, top) 2.4 (●), 3.4 (▽), and 5.0 μM (▼) or (B, bottom) 8.6 (●), 151.1 (▽), and 30.8 μM (▼) assembly-competent tubulin. Nucleotide-depleted microtubule protein at the stated concentrations was assembled with 100 μM GTP and a GTP-regenerating system. The lines are linear least-squares fits to the data to yield values of $k_{+1,GTP}$[M].

The nonlinearity of $k_{+1,GTP}$[M] *vs* [M] (Figure 2) is surprising since log [M] is, as predicted, proportional to log [Tu^{GTP}] (Figure 1) at somewhat lower tubulin concentrations. This nonlinearity implies that $k_{+1,GTP}$ varies as a function of [Tu^{GTP}]. Indeed, the assembly of increasing concentrations of nucleotide-depleted microtubule protein with 100 μM GTP shows that $k_{+1,GTP}$[M] increases to a maximum at about 17.5 μM tubulin of about 10×10^{-3} s^{-1} (Figure 4), even though $k_{+1,GTP}$[M] would be predicted, at this protein concentration, to exceed 20×10^{-3} s^{-1}.

It was not possible to analyze the GTP-induced polymerization at concentrations above ~25 μM tubulin as the protein assembled too rapidly. This was due in part to the efficiency of self-nucleation, but also to the very high association rate constant ($k_{+1,GTP}$) for this protein in the NaCl-supplemented buffer: 40×10^6 M^{-1} s^{-1} *vs* approximately 10×10^6 M^{-1} s^{-1} under other buffer conditions [Burns (1991a) and Symmons and Burns (1991) *vs*, for example, Johnson and Borisy (1977), Farrell and Jordan (1982), Rothwell et al. (1986), and Walker et al. (1988)]. It has, however, been possible to inspect the effect of protein concentration in greater detail by promoting polymerization with 2′dGTP, a nucleotide triphosphate which enhances self-nucleation but suppresses the subsequent elongation (Hamel et al., 1984).

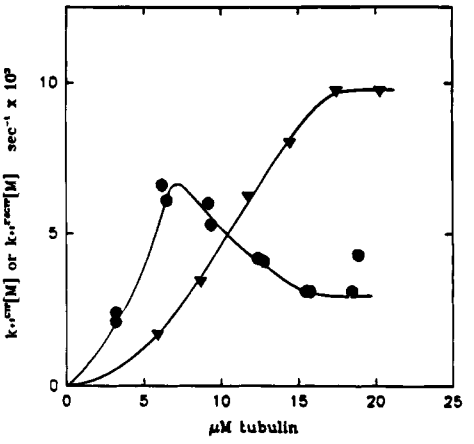

FIGURE 4: Pseudo-first-order rate constants $k_{+1,2'dGTP}[M]$ and $k_{+1,GTP}[M]$ as a function of the concentration of assembly-competent tubulin. Increasing concentrations of nucleotide-depleted microtubule protein were assembled with 100 μM 2'dGTP or 100 μM GTP. Values of $k_{+1,2'dGTP}[M]$ (●) or $k_{+1,GTP}[M]$ (▼) were derived as described in Figures 2 and 3.

Increasing concentrations of nucleotide-depleted microtubule protein were assembled with 100 μM 2'dGTP. Unlike the GTP-induced assembly, the pseudo-first-order plots were nonlinear at higher initial protein concentrations. These plots were concave, with the instantaneous rate of subunit addition during the later phase of elongation *exceeding* that predicted from the earlier elongation phase. This contrasts with the convex curvature in the pseudo-first-order plots immediately before the onset of steady state for the GTP-induced assembly, and which is a direct consequence of dynamic instability (Symmons & Burns, 1991). Values of $k_{+1,2'dGTP}[M]$ were therefore calculated from the earlier phase of the assembly. They increased to a maximum at an initial protein concentration of about 6 μM tubulin, but decreased at higher concentrations (Figure 4).

The apparent value of $K_{+1,2'dGTP}$ for assembly at 6 μM tubulin was determined by relating [M], from the lengths of negatively stained microtubules and the observed extents of assembly, with the observed values of $k_{+1,2'dGTP}[M]$. This approach yielded an apparent value of less than 10×10^6 M^{-1} s^{-1} (data not shown), *i.e.*, significantly lower than $k_{+1,GTP}$. The maximum value of $k_{+1,2'dGTP}[M]$ (6×10^{-3} s^{-1} at 6 μM tubulin, Figure 4) is therefore due to the enhanced self-nucleation (>600 pM microtubules compared with about 31 pM for microtubules assembled with GTP at the same protein concentration). These studies therefore confirm the earlier report (Hamel et al., 1984) that 2'dGTP enhances nucleation but suppresses elongation.

We have examined the assembly by 2'dGTP in greater detail by determining the maximum extent of assembly of nucleotide-depleted microtubule protein (20 μM tubulin) as a function of the 2'dGTP concentration (Figure 5). Surprisingly, exceptionally low (<5 μM 2'dGTP) concentrations efficiently promoted microtubule assembly, as confirmed by negative-stain electron microscopy. Under these conditions, approximately 25% of the tubulin had 2'dGTP bound to the β-subunit exchangeable site and 25% had bound GDP, while half had no bound nucleotide. Clearly, the 2'dGTP-induced assembly was not prevented by either the unliganded tubulin or the Tu^{GDP}. The maximum extent of assembly slightly exceeded the 2'dGTP concentration, although this may have been an artifact since the formation of a small number of

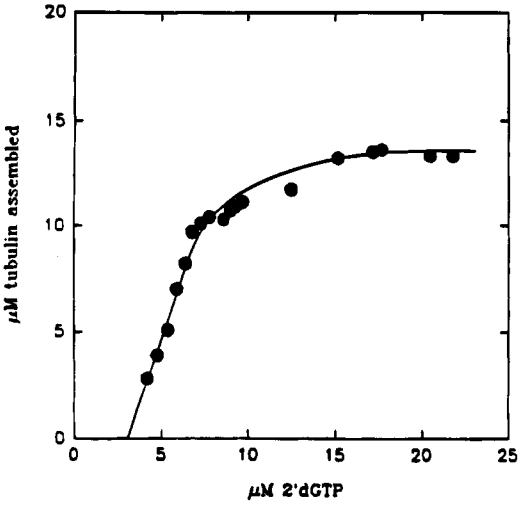

FIGURE 5: Maximum extent of microtubule assembly as a function of the 2'dGTP concentration. Nucleotide-depleted microtubule protein (2.5 $mg \cdot mL^{-1}$, 20 μM tubulin) was assembled with increasing 2'dGTP concentrations.

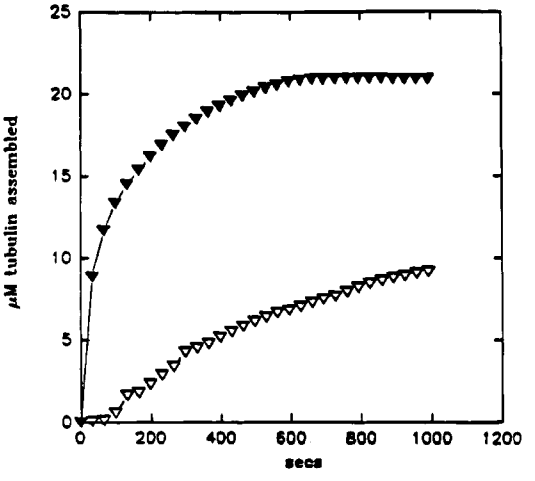

FIGURE 6: Kinetics of assembly of nucleotide-depleted microtubule protein (3.16 $mg \cdot mL^{-1}$, 25.3 μM tubulin) with (▽) 10 or (▼) 100 μM 2'dGTP.

sheets or other polymorphs, including, for example, aggregates of unliganded tubulin, could account for the turbidometric overshoot.

This polymerization with substoichiometric 2'dGTP concentrations permits an examination of nucleation as function of $[Tu^{2'dGTP}]$ rather than of the total tubulin concentration. Nucleotide-depleted microtubule protein (25.3 μM tubulin) was assembled with 10 or 100 μM 2'dGTP (Figure 6). Samples were taken for negative-stain electron microscopy shortly before steady state was attained to determine [M] from the mean microtubule lengths and the extents of assembly: 1.78 nM microtubules were assembled at 100 μM 2'dGTP and 257 pM at 10 μM 2'dGTP. These, with the observed values of $k_{+1,2'dGTP}[M]$, yield estimates of $k_{+1,2'dGTP}$ of 4.35×10^6 and 6.15×10^6 M^{-1} s^{-1}, respectively. This method of determining the association rate constant from the pseudo-first-order plot, the extent of assembly, and the mean microtubule length immediately before the onset of steady state has yielded reproducible values for $k_{+1,GTP}$ [$(40 \pm 2) \times 10^6$ M^{-1} s^{-1}]. The nonlinearity of the 2'dGTP pseudo-first-order plots, particularly at high initial protein concentrations, indicates that the observed values of $k_{+1,2'dGTP}$

(*i.e.*, 6.15×10^6 and 4.35×10^6 M^{-1} s^{-1}) may not be significantly different (see Discussion).

DISCUSSION

Several lines of evidence indicate that the early stages of the assembly of chick brain microtubule are not fully described by the simple models for nucleation and the subsequent elongation toward steady state (see the introduction). These include the apparent dependency of $k_{+1,GTP}$ on the tubulin concentration [Figure 2; and see also Engelborghs et al. (1977) and Carlier and Pantaloni (1978)], the suppression of $k_{+1,GTP}$[M] on adding excess seeds, and the enhanced efficiency of nucleation by 2′dGTP compared with GTP. Furthermore, the pseudo-first-order plots (*e.g.*, Figure 3) for GTP-induced assembly are linear from shortly after the temperature-jump until immediately before steady state. This demonstrates that there is no additional nucleation during the elongation phase, even though the free [Tu^{GTP}] can exceed a concentration which is sufficient to effect nucleation (compare the pseudo-first-order plots for the assembly of 2.4 and 30.8 μM tubulin; Figure 3, top and bottom panels). This distinction between nucleation and elongation is further demonstrated by an analysis of the microtubule length distributions as a function of the elongation time (Symmons & Burns, 1991): there is minimal nucleation during the elongation phase. There is consequently substantial evidence that nucleation differs significantly from the subsequent elongation.

One possible cause might be that the tubulin oligomers, which are detected as a major product of microtubule disassembly (Lange et al., 1988; Obermann et al., 1990; Mandelkow et al., 1988, 1991), could participate differently in nucleation and elongation. Indeed, the dissociation of these oligomers and/or the subsequent GTP−GDP exchange reaction have been postulated to be the kinetically slow process underlying the observed time-dependent oscillations in the extent of microtubule assembly (Melki et al., 1988; Caplow & Shanks, 1990). The potential role of such oligomers can, however, be eliminated since no oligomers can be detected under the current buffer conditions (Burns, 1991a). Furthermore, the oligomers implicated in the oscillations are only present above a critical concentration of about 20−25 μM tubulin (Melki et al., 1988), while the rate constant for oligomer dissociation is $(20-45) \times 10^3$ s^{-1} (Melki et al., 1988; Caplow & Shanks, 1990).

Previous studies of the temperature modulation of nucleation have indicated that tubulin undergoes a preassembly conformational change (Carlier, 1983). This change could occur either in free solution or in the terminal subunit of an elongating microtubule prior to the acceptance of an additional incoming subunit. These two alternatives are formally equivalent to the two schemes considered when examining the assembly of Tu^{GDP} subunits by taxol and Taxotere (Diaz et al., 1993). In the first model, the unassembled subunits would initially be in the $Tu^{1,GTP}$ (or $Tu^{1,2'dGTP}$) conformation, would change in free solution to the $Tu^{2,GTP}$ (or $Tu^{2,2'dGTP}$) conformation with first-order kinetics, and would then be added to the microtubule end with the pseudo-first-order kinetics determined by $k_{+1,GTP}$[M] and $Tu^{2,GTP}$ (or $k_{+1,2'dGTP}$[M] and $Tu^{2,2'dGTP}$). Such a model would predict a lag phase before the onset of elongation. However, nucleation and the conditions of the temperature jump would also induce a lag phase. Consequently, while a lag phase is observed (the maximum rate of elongation is observed approximately 30 s after the initiation of assembly), it cannot be ascribed solely to the proposed conformational change. The second model is different in that it assumes that the conformational change occurs after subunit addition to the microtubule end. The Tu^{GTP} of the microtubule end could therefore exist in two states: $Tu^{1,GTP}$ (or $Tu^{1,2'dGTP}$) would be added with pseudo-first-order kinetics to those microtubules ending with a subunit in the $Tu^{2,GTP}$ (or $Tu^{2,2'dGTP}$) state. This addition would convert the microtubule end to the $Tu^{1,GTP}$ (or $Tu^{1,2'dGTP}$) state, preventing any further subunit addition until this terminal subunit had undergone the first-order conformational change (*i.e.*, $Tu^{1,GTP}$ or $Tu^{2,GTP}$).

Unfortunately, the elongation kinetics do not formally distinguish between these alternatives, nor exclude a third possibility, namely, that conformational change can occur both in the free subunit and following addition to the microtubule end. The first model, namely, that the conformational change occurs in free solution, is supported by the assembly at low 2′dGTP concentrations (Figures 5 and 6). In particular, Tu^{GDP} and unliganded subunits do not affect the observed association rate constant (4.35×10^6 *vs* 6.15×10^6 M^{-1} s^{-1}, Figure 6), which suggests that they fail to bind significantly to the microtubule end, while fast assembly was observed at even the lowest 2′dGTP concentrations (Figure 5). The alternative possibility, namely, that the conformational change occurs following subunit addition to the microtubule end, cannot be excluded. It would, however, be necessary to presume that the terminal subunit of an elongating microtubule has a much higher affinity for a nucleotide triphosphate than the free subunits in order to account for the assembly kinetics at substoichiometric 2′dGTP concentrations.

One way of producing a sharp boundary between nucleation and elongation would be to specify (a) that the conformational change can occur in free solution, (b) that only subunits in the $Tu^{1,GTP}$, or $Tu^{1,2'dGTP}$, conformation can participate in nucleation, and (c) that subunits in the $Tu^{2,GTP}$, or $Tu^{2,2'dGTP}$, conformation are only used during elongation (see Figure 7). The abrupt cessation of nucleation during the GTP-induced assembly would therefore reflect the time-dependency of the conformational change. This model is supported by the marked dependency of nucleation on the rate of a 0−37 °C temperature-jump (Obermann et al., 1990), by the selective inhibition of elongation by colchicine (Lambeir & Engelborghs, 1983), and by the differing efficiencies of various GTP analogs to support nucleation. In particular, there is no nucleation in the presence of GTPγS even though this analog supports the subsequent elongation (Symmons & Burns, 1991), while certain guanine triphosphates (*e.g.*, Figure 4; Hamel et al., 1984) are more effective than GTP in nucleating assembly but less effective at supporting elongation.

The structural nature of this change is unknown. While the protein prior to the initiation of assembly has GDP bound to the exchangeable site on the β-tubulin, it is unlikely that the conformational change simply reflects GDP−GTP or GDP−2′dGTP exchange since the binding is rapid (0.11−0.2 s^{-1}; Engelborghs & Eccleston, 1982; Melki et al., 1988; Caplow & Shanks, 1990). The conformational change may, however, occur in response to this nucleotide exchange, as it is temperature-dependent (Carlier, 1983; Obermann et al.,

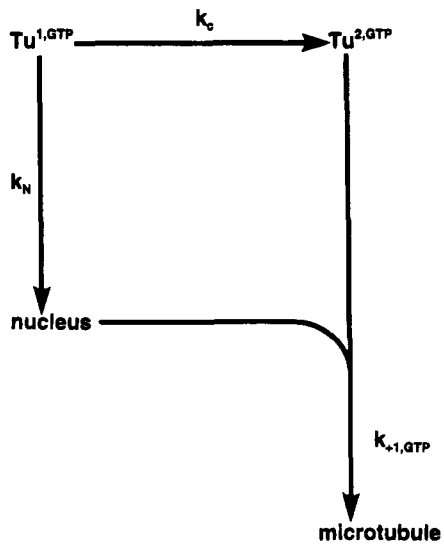

FIGURE 7: Schematic of the early events of GTP-induced microtubule assembly. Two conformational states ($Tu^{1,GTP}$ or $Tu^{2,GTP}$) are shown, related by a first-order rate constant, k_c. The $Tu^{1,GTP}$ conformational state assembles, with a rate constant k_N, to form a nucleus. The number of tubulin dimers involved in nucleus formation is unknown, but is likely to be less than the experimentally determined value of 4−5 subunits (see Discussion). Elongation occurs by the addition of $Tu^{2,GTP}$ subunits to this nucleus. For simplicity, the rate constant governing this addition is shown as $k_{+1,GTP}$, *i.e.*, the rate constant governing the addition of $Tu^{2,GTP}$ subunits to a microtubule end. Only the forward reactions are shown.

1990). Consequently, the conformational change may represent the reverse of that associated with the assembly-dependent GTP hydrolysis which results in the behavior of dynamic instability. Indeed, the binding of GTP or a GTP analogue has been postulated to alter the conformation of the unassembled subunit from a "curved" state, which results in the formation of the oligomeric rings characteristic of the microtubule cold-dissociation product, to a "straight" state, which is capable of being added to the microtubule end (Melki et al., 1989). Conversely, the assembly-dependent GTP hydrolysis alters the conformation from the "straight" to the "curved" form, with the consequential weakening of the subunit−subunit interaction and enhancement of the dissociation rate constant ($k_{-1,GTP} \ll k_{-1,GDP}$). The switch between these two conformational states may specifically involve the metal-coordinated occupation of the γ-phosphate binding site (Shearwin & Timasheff, 1992). This model of "curved" and "straight" states, which has been extended to the taxol-induced assembly of Tu^{GDP} (Diaz et al., 1993), may correspond to the conformational states associated with nucleation (*i.e.*, $Tu^{1,GTP}$ may be the "curved" state) and elongation (*i.e.*, $Tu^{2,GTP}$ may be the "straight" state). The conformational change would therefore be induced at the elevated temperature required for microtubule assembly by NTP binding (*e.g.*, 2′dGTP, GTP, and GTPγS). The differences between the nucleotides in the promotion of nucleation, relative to the support of elongation, demonstrate that the value of k_c is NTP-specific.

The dependency of [M] on the protein concentration (n = 2.25, Figure 1) would conventionally imply that the functional nucleus involves the interaction of four to five subunits. This compares with the value of n = 2 for the assembly of unfractionated microtubule protein or the DMSO-induced assembly of pure tubulin (Engelborghs et al., 1977; Robinson & Engelborghs, 1982). Significantly, this earlier analysis failed to take account of nucleation occurring even when the $[Tu^{GTP}]$ is less than the elongation critical concentration; *i.e.*, a plot of [M] *vs* $[Tu^{GTP}]$ must pass through the origin. Replotting their data obtained at low (below about 20 μM) tubulin concentrations as $\log(k_{+1,}{}^{GTP}[M])$ *vs* log $[Tu^{GTP}]$ yields slopes of 2.64 for the nucleation of unfractionated microtubule protein and 1.87 for the DMSO-induced assembly of pure tubulin. Conversely, the observed value of K_n (2.25, Figure 1) is much lower than that previously reported for the assembly of pure tubulin in 3.4 M glycerol/5 mM $MgCl_2$ (10−12; Carlier & Pantaloni, 1978). Replotting their data as $\log(k_{+1,GTP}[M])$ *vs* log $[Tu^{GTP}]$ for nucleation below about 15 μM tubulin yields a slope of 3.4; *i.e.*, nucleation apparently involves the interaction of six to eight subunits. A lower value (four to five dimers) has been reported, using a kinetic approach to evaluate the minimum nucleation particle, for the assembly of pure tubulin with 1.4 M dimethyl sulfoxide (Algaier & Himes, 1988). Such variations in the value of n are unexpected, even though differing buffer conditions have been used. Furthermore, the detailed analysis using an approach based upon the time to achieve 10% maximal assembly (Algaier & Himes, 1988) found an unexpected variation in the apparent number of dimers required for nucleation, even though the assembly was studied under controlled conditions.

The conclusion that only the $Tu^{1,GTP}$ (or the $Tu^{1,2'dGTP}$) conformation participates in nucleation not only offers a mechanistic explanation for the rapid termination of nucleation but also accounts for the apparent variation in the number of subunits required for nucleation, and indicates that the $[M] = K_n[Tu^{GTP}]^n$ relationship is flawed. The current work shows that it is necessary to estimate the instantaneous $Tu^{1,GTP}$ concentration. No study has yet determined the true number of subunits required to form an effective nucleus, although the true number is likely to be lower than the various published values. This criticism applies to all the studies, irrespective of the initial protein concentration, since it is necessary to plot log [M] *vs* log $[Tu^{1,GTP}]$ rather than against log $[Tu^{GTP}]$. Indeed, the conclusion that $Tu^{1,GTP}$ is required for nucleation while $Tu^{2,GTP}$ participates in elongation greatly complicates any analysis of the earliest stages of microtubule assembly (Figure 7). The efficiency of nucleation will involve the rate constant k_N and an unknown function of the instantaneous $[Tu^{1,GTP}]$. Similarly, elongation will depend upon $[Tu^{2,GTP}]$. This is also a time-dependent concentration, equaling $k_c t[Tu^{1,GTP}]$ less the instantaneous extent of microtubule assembly. The rate constant k_c which governs the $Tu^{1,GTP}$ to $Tu^{2,GTP}$ conformational change is unknown, but with increasing time, all of the subunits will have adopted the $Tu^{2,GTP}$ conformation. This is confirmed by the linearity of the pseudo-first-order plots (*e.g.*, Figure 3), which furthermore indicates that the conformational change, for assembly with GTP, is effectively complete in less than 1 min from the temperature-jump. By contrast, the value of k_c appears to be significantly smaller for the assembly with 2′dGTP: there is far higher nucleation at equivalent protein concentrations with 2′dGTP than with GTP, and the pseudo-first-order plots are concave.

Two factors complicate the analysis of the 2′dGTP-induced assembly. First, the low apparent value of k_c means (see Figure 7) a less abrupt transition between nucleation and elongation than for the GTP-induced assembly. This would

yield the concave pseudo-first-order plot, due to both the continuing nucleation with time of additional microtubules *and* the continuing production of $Tu^{2,2'dGTP}$. The second complication arises from the low apparent value of $k_{+1,2'dGTP}$ [$(4-6) \times 10^6$ M^{-1} s^{-1}]. Dynamic instability alters the elongation kinetics when the instantaneous rate of NTP hydrolysis equals or exceeds the instantaneous rate of subunit addition (*i.e.*, $k_{+1,2'dGTP}Tu^{2,2'dGTP}$). This corresponds, for GTP-induced assembly under the current assembly conditions, to approximately 200 subunits s^{-1} (Symmons & Burns, 1991); the equivalent value for 2'dGTP-induced assembly is unknown. This effect results in a *convex* pseudo-first-order plot (Symmons & Burns, 1991). The observed concave plots may, however, mask an early onset of dynamic instability. This could contribute, together with the kinetics of the $Tu^{1,2'dGTP}$ to $Tu^{2,2'dGTP}$ conformational change, to the observed decline in $k_{+1,2'd}{}^{GTP}$[M] above an initial concentration of about 6 μM tubulin (Figure 4).

Finally, the effective nucleus must also initiate the assembly-dependent NTP hydrolysis under those conditions which permit the formation of a detectable "GTP-cap" [see Carlier et al. (1987) and Burns (1991b)], *i.e.*, those conditions which permit the docking of an additional $Tu^{2,GTP}$ subunit to the microtubule end without the consequential induction of GTP hydrolysis in the subterminal subunit. The proposal that the functional nucleus is formed from subunits in the $Tu^{1,GTP}$ conformation, *i.e.*, in the conformation which appears to correspond to that adopted following the assembly-dependent GTP hydrolysis, offers an attractive mechanism for initiating this process.

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