Assembly of Pure Tubulin in the Absence of Free GTP: Effect of Magnesium, Glycerol, ATP, and the Nonhydrolyzable GTP Analogues†

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ABSTRACT: We describe in vitro microtubule assembly that exhibits, in bulk solution, behavior consistent with the GTP cap model of dynamic instability. Microtubules assembled from pure tubulin in the absence of free nucleotides could undergo one cycle of assembly, but could not sustain an assembly plateau. After the initial peak of assembly was reached and bound E-site GTP hydrolyzed to GDP, the microtubules gradually disassembled. We studied buffer conditions that maximized this disassembly while still allowing robust assembly to take place. While both glycerol and glutamate increased the rate of initial assembly and then slowed disassembly, magnesium promoted initial assembly and, surprisingly, enhanced disassembly. After cooling, a second cycle of assembly was unsuccessful unless GTP or the hydrolyzable GTP analogue GMPCPOP was readded. The nonhydrolyzable GTP analogues GMPPNP and GMPPCP could not support the second assembly cycle in the absence of E-site GTP. Analysis using HPLC found no evidence that GMPPNP, GMPPCP, or ATP could bind to free tubulin, and these nucleotides did not compete with GTP for the E-site. We have, however, demonstrated that the nonhydrolyzable GTP analogues and ATP do have an important effect on microtubule assembly. GMPPNP, GMPPCP, and ATP could each enhance the rate of assembly and stabilize the plateau of assembled microtubules against disassembly, while not binding appreciably to free tubulin. We conclude that these nucleotides, as well as GTP itself, enhance assembly by binding to a site on microtubules that is not present on free, unpolymerized tubulin. We estimate the affinity (K_D) of the polymeric site for nucleotide triphosphates to be approximately 10^{-4} M.

Soon after a tubulin subunit is incorporated into a microtubule polymer, one GTP[1] molecule is hydrolyzed to GDP and free phosphate (Kobayashi, 1975; Weisenberg et al., 1976; Arai & Kaziro, 1976; David-Pfeuty et al., 1978; MacNeal & Purich, 1978). These studies also showed that the GTP hydrolyzed during assembly had been tightly bound to free tubulin at the exchangeable binding site (E-site) before assembly [defined by Weisenberg et al. (1968)]. Although these facts have been firmly established, the exact role that GTP hydrolysis plays in microtubule assembly is still not understood.

The observation that assembled microtubules specifically bound only guanine nucleotides (Weisenberg et al., 1968; Yanagisawa et al., 1968), coupled with the knowledge that GTP was hydrolyzed during assembly, seemed to indicate that GTP hydrolysis was necessary for microtubule assembly. However, experiments showing assembly in the presence of the nonhydrolyzable GTP analogues GMPPNP and GMPPCP implied that, although GTP was required for assembly, hydrolysis to GDP and free phosphate was not required (Weisenberg et al., 1976; Arai & Kaziro, 1976; Penningroth & Kirschner, 1977). It was therefore suggested that hydrolysis, rather than being necessary for assembly, might be necessary to allow rapid disassembly of microtubules (Weisenberg et al., 1976; Arai & Kaziro, 1976) and also that GTP hydrolysis need not be directly coupled to assembly, but could lag behind (MacNeal & Purich, 1978). Evidence for such a lag [later shown to have been greatly overestimated (O'Brien et al., 1987)] was presented by Carlier and Pantaloni (1981) in simultaneous measurements of GTP hydrolysis and microtubule assembly. These ideas were later extended by Mitchison and Kirschner (1984) as part of the "GTP cap" model explaining dynamic instability. The proposal that, under defined buffer conditions, most microtubules in a steady-state population are slowly growing while a few are rapidly disassembling is nicely explained by the idea that microtubules are composed of a labile GDP–tubulin core stabilized by caps of GTP–tubulin. This hypothesis, with slight modifications, remains the most attractive explanation advanced thus far to explain these dynamic properties.

We began the present work to explore in more detail the properties of microtubules assembled with GMPPNP and GMPPCP, in the absence of E-site GTP and contaminating MAPs. Microtubules assembled with the analogues should exhibit the properties of GTP caps, i.e., be more resistant to calcium ion (Weisenberg et al., 1976; Arai & Kaziro, 1976; Penningroth & Kirschner, 1977), have an appreciably lower critical concentration for assembly (Jameson & Caplow, 1980), should not show dynamic instability behavior, and would perhaps undergo a greater frequency of annealing. We used a cycle of glutamate assembly after phosphocellulose chromatography to eliminate any remaining associated proteins and non-tubulin enzymatic activity from our tubulin preparation (Voter & Erickson, 1984; O'Brien et al., 1987), and a combination of gel filtration and a cycle of assembly/disassembly to remove all GTP from tubulin without removing N-site GTP or denaturing the tubulin. We measured assembly

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GDP, guanosine 5′-diphosphate; GMPPCP, guanylyl (β,γ-methylene)diphosphonate; GMPPNP, guanylyl imidodiphosphonate; GMPCPOP, guanosine 5′-(α,β-methylene)triphosphate; GTP, guanosine 5′-triphosphate; HPLC, high-pressure liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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and the concentration of total nucleotide spectrophotometrically, and determined the identity of the nucleotides present using HPLC. After gel filtration, the tubulin could undergo one cycle of assembly but could not maintain a stable plateau without free GTP and could not assemble a second time.

Our results indicate that the nonhydrolyzable GTP analogues do not support microtubule assembly in the absence of E-site GTP, and, surprisingly, do not measurably interact with the E-site of tubulin. However, the analogues did enhance the rate of assembly of GTP–tubulin in the absence of free GTP and stabilized the peak of assembly against spontaneous disassembly. Interestingly, these effects were also observed, at exactly the same concentrations, with ATP. In order to study these effects, we explored buffer conditions that maximized both assembly and disassembly during the first cycle of assembly after removal of free GTP, characterizing important effects of glycerol and magnesium ion on the assembly process. Our results indicate that the analogues and ATP enhance assembly via an interaction with a binding site on the polymeric form of tubulin, and may help to reconcile some of the conflicting reports concerning the assembly-enhancing properties of both the nonhydrolyzable GTP analogues and ATP. Since the analogues did not bind to the E-site under our conditions, and hydrolysis takes place rapidly after subunit addition (O'Brien et al., 1987; Walker et al., 1988), we may now need to reopen the following question: when GTP is present at the E-site, is hydrolysis necessary for that subunit to assemble past one layer in depth?

Experimental Methods

Porcine brain tubulin was purified and freed of MAPs as described previously (Voter & Erickson, 1984) and stored at −80 °C in a buffer consisting of 50 mM Mes, 1 mM EGTA, 0.5 mM $MgSO_4$, 3.4 M glycerol, and 0.5 mM GTP, pH 6.6. On the day of an experiment, tubulin was freed of unbound nucleotides by passage (at 4 °C) over a Sephadex G-25 column (Pharmacia PD-10) equilibrated with the buffer needed for a particular experiment, in the cold. This tubulin was designated "minus free nucleotide" tubulin, or −fT tubulin. Typically, 0.3–0.6 mL of tubulin (6–8 mg/mL) was added to the Sephadex column and 0.6–0.9 mL collected. For experiments varying a particular buffer component, the column was equilibrated with a buffer containing a minimum concentration of that component and then additional amounts were added before the experiment was begun, or, with a high enough concentration of tubulin, a component could be diluted to the desired final concentration after passage through the column.

Microtubule assembly at 37 °C was monitored by turbidity and checked by warm centrifugation of aliquots from the assembly reaction, as described previously (O'Brien et al., 1987). Similarly, the total quantity of nucleotide present in samples of tubulin was determined spectrophotometrically after precipitation of the tubulin, and the relative concentrations of each nucleotide were determined by using HPLC, also as described previously (O'Brien et al., 1987).

The binding of nucleotides to polymerized microtubules was determined under equilibrium conditions. Tubulin (2.5 mg/mL, −fT), in the Mes–glycerol buffer described above with 5 mM $MgSO_4$ added (MEMG assembly buffer), was assembled at 37 °C for 15 min in the presence of either 500 μM GTP, ATP, or GMPPNP or without added nucleotides. A trace amount of tritiated water was also added to each sample. The microtubules were then centrifuged for 18 min at 30 000 rpm in a Beckman Type 50 rotor, the supernatants removed, and the pellets resuspended in 0.02 M NaOH. After an aliquot was removed from each sample for protein determination (Bradford protein assay, Bio-Rad Laboratories, purified tubulin standard) and for evaluation of radioactivity, the remainder of each sample was prepared for spectral analysis of nucleotide concentration and HPLC as described previously (O'Brien et al., 1987). The tritium tracer allowed calculation of the pellet volumes and thus the amounts of free nucleotide expected trapped in the pellets.

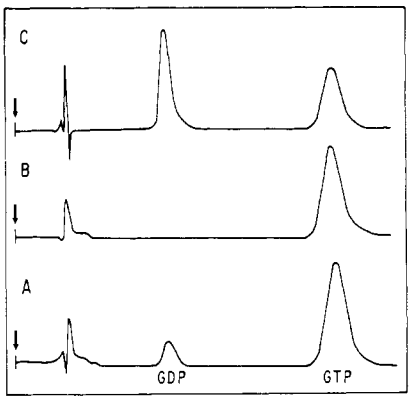

FIGURE 1: HPLC traces of the guanine nucleotides present before (A) and after (B) passage through a Sephadex column and 1 min after assembling to a peak (C).

GMPPNP and GMPPCP were obtained from Boeringer Mannheim, GMPCPOP was obtained from ICN, and all other nucleotides were obtained from Sigma Biochemicals.

Results

We prepared tubulin free of unbound nucleotide by passage over a Sephadex G-25 column in the cold. This was sufficient to remove all unbound nucleotide. HPLC and spectrophotometric techniques were used to determine the quantity and identity of the nucleotides present before and after passage through the column, as well as after a peak of assembly had been reached (Figure 1). Before passage through the Sephadex column, about 10% of the total nucleotide present was GDP, and the total concentration of nucleotide (bound and free) was about 500 μM (Figure 1A). Collecting only a narrow region of the protein peak prevented measurable free nucleotide from eluting with the tubulin. Figure 1B shows the nucleotide present after passage through the Sephadex column. No GDP peak was seen, and all of the nucleotide present was GTP. The concentration of tubulin collected from the Sephadex column (on the basis of a molecular weight of 100 000) was 25.7 μM, and that of total nucleotide was 51.8 μM, representing 2 mol of GTP/mol of tubulin. Thus, no appreciable nucleotide was present that was not bound to tubulin.

Passage over the Sephadex column did not affect the ability of tubulin to assemble. At a tubulin concentration of 2.5 mg/mL, in MEMG assembly buffer (50 mM MES, 1 mM EGTA, 5 mM $MgSO_4$, 3.4 M glycerol), with 0.3 mM GTP readded to the tubulin before warming, the tubulin assembled normally to a plateau within 10 min (Figure 2, unbroken line). Under the same conditions, tubulin without free GTP present ("minus free GTP" or −fT tubulin) assembled somewhat more slowly and did not sustain a stable plateau of assembly (Figure 2, dashed line). Rather, after reaching a peak the concentration of polymer declined slowly. The apparent decline in polymer mass indicated by absorbance measurements was confirmed by sedimenting aliquots of the reaction mixture into stabilization buffer (MEM buffer with 10% DMSO and 1% glutaraldehyde added) and evaluating the polymer mass after centrifugation (data not shown). This assay also confirmed that there was no decline in polymer mass when GTP was

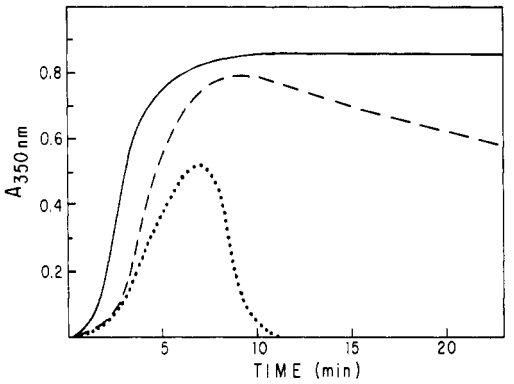

FIGURE 2: Assembly of tubulin in the presence of GTP or freed of unbound nucleotide. Tubulin, freed of unbound nucleotide by passage through a Sephadex column, was assembled at 37 °C under two different buffer conditions or with 300 μM GTP added. (Solid line) GTP readded, magnesium glycerol buffer, 2.5 mg/mL tubulin. (Dashed line) No free nucleotide, magnesium glycerol buffer, 2.5 mg/mL tubulin. (Dotted line) No free nucleotide, 6 mg/mL tubulin, Pipes, glycerol-free buffer.

present. Negative stain electron microscopic evaluation of the polymers formed, with or without free GTP present, showed a mixture of long narrow sheets with a clearly evident protofilament structure, as well as microtubules. For convenience we will refer to this mixture of microtubules and ribbons as "microtubules" or "polymers". The significance of non microtubule polymers in assembly with glycerol is discussed in the Discussion.

For comparison with assembly in glycerol, -fT tubulin was prepared in Pipes buffer (PB) without glycerol, the buffer in which dynamic instability of microtubules was first described (Mitchison & Kirschner, 1984). In PB, much higher tubulin concentrations were required to achieve self-nucleated assembly. Figure 2 (dotted line) illustrates the assembly of 6 mg/mL -fT tubulin. Although the peak of assembly was obtained at about the same time as that observed in MEM buffer, assembly in PB showed almost no plateau phase. Instead, the microtubules appeared to disassemble very quickly after reaching a peak.

During microtubule assembly, GTP hydrolysis takes place very soon after, or concurrently with, incorporation of a tubulin subunit into a microtubule, both in MEM and in PB (O'Brien et al., 1987). Thus, GTP caps present at the peak of assembly must be quite short or absent. The lack of a stable plateau observed with -fT tubulin is consistent with the idea that, with no exogenous GTP to replenish the GTP caps at the ends of each microtubule, caps remaining at the peak of assembly are gradually lost, either by spontaneous hydrolysis (Carlier & Pantaloni, 1981) or through an intrinsic off-rate. As the caps are lost, individual polymers should begin to disassemble, yet microtubules in the glycerol buffer (MEMG) showed much slower disassembly than in the PB after a peak of assembly was reached.

To corroborate that the relatively slow disassembly in MEM was not due to the presence of large numbers of GTP–tubulin dimers in the microtubules just after the peak of assembly, we removed a sample from the microtubule mixture at 10 min after the beginning of assembly (Figure 2, dashed line). The sample was then immediately processed for HPLC as described in the caption to Figure 1. The result is shown in Figure 1C. Again, the total nucleotide corresponded to about 2 mol of GXP/mol of tubulin (within 1–4%), but this time approximately half the nucleoide present was GDP (in this example, 49.8%). Although not all the tubulin present was in the polymer form at the peak of polymerization (with 25 μM total

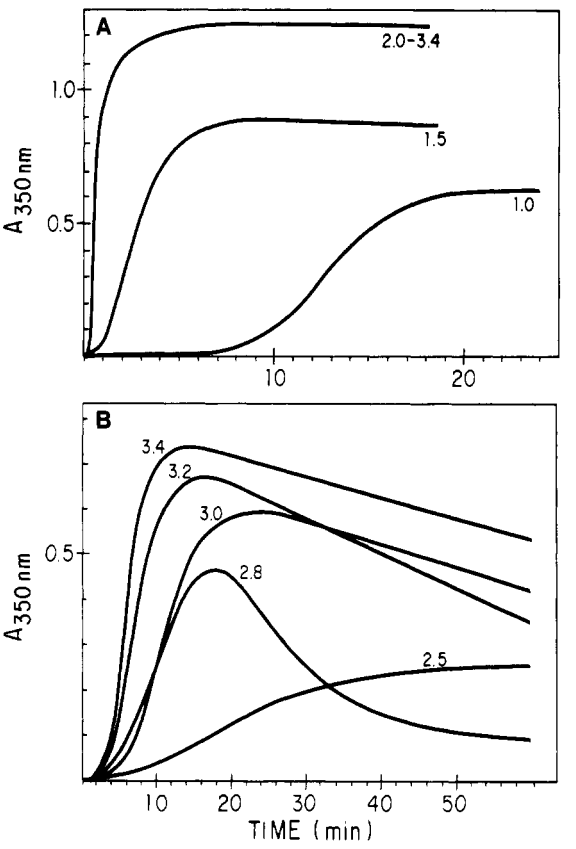

FIGURE 3: Assembly of 2.5 mg/mL tubulin in MEMG buffer (5 mM $MgSO_4$) with various concentrations of glycerol in the presence (A) or absence (B) of free GTP. Numbers indicate the molar concentration of glycerol used.

tubulin, 60–70% of the tubulin was polymer at the peak of assembly), apparently most of the dimers present had passed through the polymer state after 10 min at 37 °C, converting 1 GTP to GDP in the process. Thus, consistent with our earlier results, essentially no E-site GTP remained bound to tubulin in either polymer or free dimer form, soon after the peak in assembly had been reached.

Glycerol or glutamate in microtubule buffers offers the advantage of protecting free tubulin from rapid denaturation, as well as allowing assembly of reasonably low concentrations of tubulin without adding assembly-promoting proteins. However, for investigations of in vitro correlates of dynamic instability, it would be advantageous to find conditions that allowed assembly of low tubulin concentrations but also allowed rapid disassembly of GDP microtubules after GTP had been exhausted. We therefore investigated changes in the MEM buffer that would maximize the apparent disassembly of -fT microtubules after the peak of assembly.

In 3.4 M glycerol, varying the concentration of Mes or Pipes from 10 to 100 mM made little difference to the rate of disassembly of -fT microtubules. Varying the concentration of EGTA from zero to 10 mM also did nothing appreciable to the rate of disassembly.

Varying the concentration of glycerol, in contrast, had a large effect, but this effect was on both assembly and disassembly. With added GTP (Figure 3A), 2.5 mg/mL tubulin assembled slowly with 1.0 M glycerol and maintained a plateau value once it had been achieved. Raising the concentration of glycerol to 1.5 M increased both the rate and extent of assembly, and a significant decrease in the lag phase of assembly was seen. At 2.0 M glycerol, the rate and extent of assembly were about twice that seen with 1 M glycerol, and the time required for nucleation of assembly was almost 10-fold

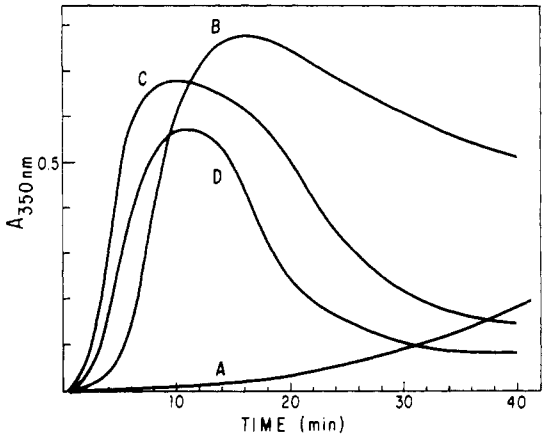

FIGURE 4: Effect of magnesium on assembly of –fT tubulin in glycerol buffer. 2.5 mg/mL pure tubulin was assembled with (A) 2.5 (A), 5 (B), 10 (C), or 15 mM (D) magnesium sulfate in Mes buffer (3.4 M glycerol).

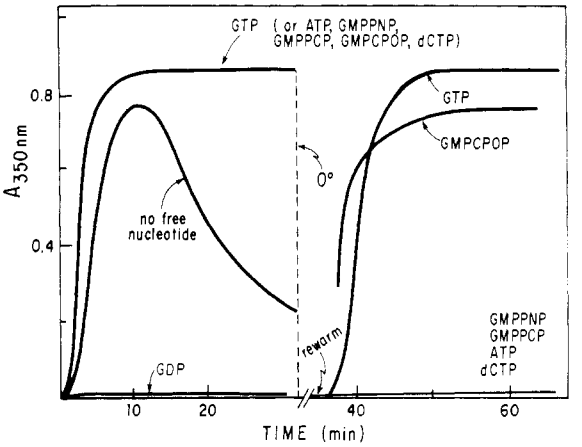

FIGURE 5: Two cycles of assembly of –fT tubulin in Mes buffer containing 2.8 M glycerol and 11 mM magnesium with various nucleotides readded. After the first assembly, the cuvette was cooled on ice for 10–15 min and then rewarmed with no further additions to the reaction solution.

less. Raising the glycerol concentration from 2.0 to 3.4 M produced no further increase in rate or extent of assembly. Thus, with excess GTP present, 2.0 M glycerol was sufficient to maximally promote assembly.

In contrast, –fT tubulin could not assemble appreciably until a concentration of 2.5 M glycerol was reached (Figure 3B). As the concentration of glycerol was decreased from 3.4 M, the disassembly rate of –fT tubulin after reaching a peak was increased slightly over that seen in buffer with higher glycerol concentrations. The effect of lowered glycerol concentration on the disassembly of –fT tubulin was not very dramatic, presumably because the bound GTP present was hydrolyzed more slowly during the slowed assembly. In the remaining experiments, we chose to use 2.8 M glycerol, a concentration that allowed somewhat faster disassembly but which was significantly above the concentration required to promote assembly of –fT tubulin.

Varying the concentration of magnesium had a dramatic effect on the assembly properties of –fT tubulin (Figure 4). Assembly of 2.5 mg/mL –fT tubulin took place more slowly as concentrations were decreased below 5 mM, and not at all below 2.5 mM magnesium. In this concentration range disassembly also was slowed as magnesium concentrations were lowered, similar to the effect of decreasing concentrations of glycerol, reflecting the slower rate of assembly.

However, as concentrations of magnesium were increased above 5 mM, assembly was promoted and the rate of disassembly was also enhanced, leading to a sharper, shorter lived peak of assembly. Above 12.5 mM, the extent and rate of assembly decreased somewhat, while the rate of disassembly was enhanced still further. In a separate experiment, we assembled –fT tubulin with 5 mM magnesium and then added 1.2% (v/v) 500 mM magnesium sulfate, resulting in a total concentration of 11 mM. The rate of disassembly increased significantly, reaching 50% of the peak height in about half the time of the 5 mM control. Thus, magnesium does not need to be present before assembly begins to affect the rate of disassembly. Magnesium was the only assembly-promoting factor we tested that also increased the rate of disassembly as concentrations were increased. To obtain the optimum balance of rapid assembly and disassembly, we used 11 mM magnesium and 2.8 M glycerol in our subsequent experiments.

If the GTP caps could be maintaned, or if assembly of GTP–tubulin could take place without GTP hydrolysis, the GTP cap hypothesis predicts the formation of stable microtubules. Thus, assembly with nonhydrolyzable GTP anlogues should inhibit the disassembly normally seen with –fT tubulin. We tested each of the GTP analogues GMPPNP, GMPPCP, and GMPCPOP, as well as GDP, ATP, ADP, AMPPNP, dCTP, and sodium phosphate, pyrophosphate, tripolyphosphate, and tetrapolyphosphate. Each was added at 2 mM to –fT tubulin on ice before the first assembly cycle. Assembly was then initiated by placing the solutions into the prewarmed cuvette and spectrophotometer (Figure 5).

The nonhydrolyzable GTP analogues, ATP, and dCTP, as well as the hydrolyzable GTP analogue GMPCPOP, enhanced the initial rate of assembly and blocked the disassembly seen without free GTP present. This was what we expected if the analogues were replacing GTP at the E-site. However, it was not expected for ATP and dCTP, which have no known affinity for the E-site (see introduction). Sedimentation of glutaraldehyde-fixed microtubules during the plateau of assembly confirmed that the apparent plateau caused by the analogues and ATP and dCTP was real, i.e., no disassembly had taken place after the peak was reached. The plateau was stable for at least 1 h at 37 °C. Negative stain electron microscopy showed the presence of ribbons and narrow sheets along with microtubules in each case, although the polymers generated in the presence of GMPCPOP were shorter and more irregular than any of the others, much like those seen in the presence of taxol. However, GMPCPOP polymers were tubulin polymers, with a protofilament structure visible. At 2 mM, the free phosphates did not block disassembly, and PP_i caused very rapid aggregation and denaturation of –fT tubulin. However, at concentrations of 25–100 mM, sodium phosphate did partially block the disassembly of the plateau, but only if added at the peak of assembly. If sodium phosphate was added before the peak was reached, disassembly was blocked, but further assembly was also immediately inhibited. If present before assembly began, these concentrations blocked assembly, a result quite different from the assembly-enhancing activity of the analogues, ATP, and dCTP. Of the nucleotides, only GDP blocked assembly of –fT tubulin, while ADP, AMPPNP, and GMP had no effect on either assembly or disassembly.

The first cycle of assembly of –fT tubulin takes place using GTP bound to the E-site, unless the nucleotide added to –fT tubulin can compete with, and thus displace, the E-site GTP. Thus, although the analogues and other nucleotides were added at a 40-fold excess relative to the GTP present, the possibility existed that the first cycle of assembly took place due only to E-site GTP still bound to tubulin. To evaluate assembly of

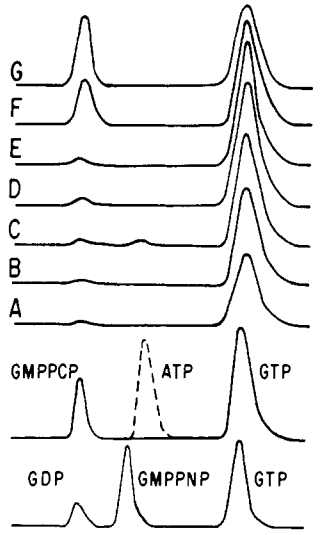

FIGURE 6: Evidence that GMPPNP, GMPPCP, and ATP have very weak affinity for the E-site of tubulin. The two bottom traces illustrate the location of peaks of standard nucleotides. The GTP peaks (A–G) represent approximately 50 μM in the original sample (0.2 mL injected). Tubulin was incubated in MEMG buffer (2.8 M glycerol, 11 mM $MgSO_4$) with (A) no added nucleotide (a small amount of GDP was present, perhaps due to spontaneous hydrolysis during the relatively long procedure) (A), 2 mM ATP (B), 10 mM ATP (C), 2 mM GMPPNP (D), 2 mM GMPPCP (E), 0.2 mM GDP (F), or 2 mM GDP (G).

–fT tubulin with no GTP present at all, we cooled microtubule solutions from the first assembly cycle to 0 °C for 15 min and then rewarmed the cuvettes without making any further additions.

The second assembly gave an unambiguous result: only GTP and the hydrolyzable GTP analogue GMPCPOP could promote a second cycle. GMPPNP, GMPPCP, ATP, and dCTP did not support the second cycle of assembly. This was not because the tubulin had denatured, since addition of GTP could stimulate assembly after 30 min of no assembly at 37 °C, in the presence of the analogues, ATP, or dCTP.

Corroborating the observation of Sandoval et al. (1977), GMPCPOP microtubules depolymerized only very slowly in the cold, and there were still a significant number of microtubules remaining after 15 min on ice. Although GMPCPOP polymers did not depolymerize fully, they did appear to disassemble somewhat, and rewarming resulted in significant new assembly that reached a stable plateau.

That GMPPNP, GMPPCP, ATP, and dCTP failed to promote the second cycle of assembly implies that they might lack a significant affinity for the E-site on tubulin. If they could bind to the E-site, GTP would have been displaced before the beginning of the first cycle, and both cycles of assembly would have been the same. Either both cycles would have been blocked, as was the case with GDP, or both would have been promoted equally. Exhaustive preincubation of tubulin with the nonhydrolyzable GTP analogues, including equilibrating the Sephadex column with analogue, did not show any signs of inhibiting the first cycle of assembly.

To assess the ability of GMPPNP, GMPPCP, and ATP to bind to the E-site of tubulin, we incubated –fT tubulin with an 80-fold excess of each nucleotide for 1 h at 4 °C and then put them through the Sephadex column a second time. We then processed each sample for HPLC to determine the amount of analogue bound or GTP displaced. The results are shown in Figure 6. For each, the total nucleotide was, as in Figure 1, very close to 2 mol of nucleotide/mol of tubulin. Two millimolar ATP, GMPPNP, or GMPPCP did not displace the 25 μM GTP from tubulin nor elute from the Sephadex column with tubulin (Figure 6B,D,E). The HPLC traces for tubulin incubated with these nucleotides were essentially identical with tubulin incubated without added nucleotide (Figure 6A). With 10 mM ATP added to the column with tubulin, a small peak of ATP did appear. However, the peak of ATP seen (Figure 6C) represents less than 0.01% of the added ATP and may be nonspecific bleed-through of the included volume. GDP, in contrast, showed a strong affinity for the E-site. Incubation with either 0.2 or 2 mM GDP showed significant binding to tubulin, with 2 mM displacing about half of the E-site GTP.

Although GMPPNP, GMPPCP, and ATP apparently cannot bind to free tubulin with high affinity, they do modulate the assembly of –fT tubulin. The block of the slow disassembly of –fT tubulin, as well as a significant and consistent increase in the rate of polymerization seen with these nucleotides (as compared to assembly of –fT tubulin), implied a possible interaction of these nucleotides with the polymer form of tubulin. One possibility is that the analogues and ATP can bind along the microtubule lattice to a "P" or polymeric binding site, as suggested by Maccioni and Seeds (1982). This, along with the E and N binding sites, would be evident as a third mole of nucleotide binding per mole of polymer assembled.

Preliminary experiments, in which steady-state microtubules with 2 mM free nucleotide present were centrifuged through 50% sucrose cushions to separate microtubules from free tubulin and unbound nucleotide, showed only 2 mol of nucleotide bound per each mole of tubulin in the pellet. However, if binding to the P-site were relatively weak, nucleotides might be released from these sites during centrifugation through sucrose. We therefore measured nucleotide binding under equilibrium conditions, where dissociation of P-site nucleotide would not take place.

To evaluate nucleotide binding under equilibrium conditions, we assembled –fT tubulin in the presence of 0.5 mM GTP, GMPPNP, or ATP or no added nucleotide and then simply centifuged the microtubules at 37 °C. We added a tritiated water tracer before centrifugation, allowing us to calculate the volume of the pellets and thus the amount of free nucleotide trapped in each. After centrifugation, both the supernatants and the resuspended pellets were precipitated and evaluated as before for total concentration of nucleotide present as well as the relative concentrations of each nucleotide. In each case, no binding to a third, stoichiometric binding site on the polymer was observed. Only 2 mol of nucleotide/mol of tubulin in polymer was found. Further, HPLC analysis was consistent with the binding of 1 mol of GDP and one of GTP per mole of tubulin in the pellet, and the concentration of GTP, ATP, or GMPPNP in the resuspended pellet was equivalent to that trapped in the pellet during centrifugation.

For example, in one experiment 350 μL of 2.5 mg/mL –fT tubulin was assembled with 585 μM ATP for 20 min and centrifuged at 37 °C (18 min, 30000 rpm, Beckman Type 50 rotor). The pellet was resuspended in 400 μL of 0.02 M NaOH. The pellet volume was calculated to be 7.7 μL, indicating that (585/407.7) × 7.7 = 11.1 μM ATP would be expected in the resuspended pellet. The resuspended pellet consisted of 13.7 μM tubulin and had a total nucleotide concentration of 36.9 μM. The 2 mol of GXP bound to the tubulin would constitute 2 × 13.7 = 27.4 μM of this total. The remaining 9.5 μM was very close to the 11.1 μM that we expected for trapped ATP. HPLC analysis, furthermore, showed that 38.2, 25.0, and 36.8% of the total nucleotide were GDP, ATP, and GTP, respectively, translating to 14.1, 9.2,

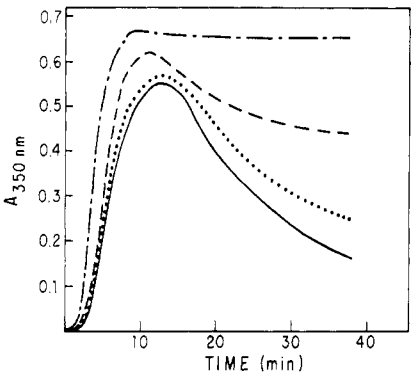

FIGURE 7: Effect of ATP on the concentration dependence of the stabilization of the microtubule assembly plateau in the absence of GTP. 25 μM −fT tubulin was assembled in 2.8 M glycerol and 11 mM magnesium buffer with no addition (solid line), 25 μM ATP (dotted line), 50 μM ATP (dashed line), or 333 μM ATP (dot/dash line) present.

and 13.6 μM of each. Thus, the ATP present can be well accounted for as that amount trapped in the pellet during centrifugation, and little ATP appears to bind to polymerized microtubules.

To assess the concentrations of nonhydrolyzable nucleotide triphosphate necessary to sustain the plateau of −fT microtubules, we added various concentrations of these nucleotides to −fT tubulin and then assembled as described previously. Using 2.5 mg/mL −fT tubulin in 2.8 M glycerol and 11 mM magnesium MEM to maximize the rate of disassembly, GMPPNP, GMPPCP, ATP, and dCTP exhibited very similar concentration–response relationships. The effect of ATP was representative and is presented in Figure 7. An approximately stoichiometric amount of ATP (25 μM) showed a slight effect, 50 μM gave about a half-maximal effect, and 300 μM consistently gave a maximum effect. If the polymer lattice is half-saturated with nucleotide triphosphate when the half-maximal effect is produced, then the affinity (K_d) of these ligands for the polymer is on the order of 10^{-4} M.

Discussion

Effects of Changes in Buffer Conditions. After free GTP was removed, the first cycle of microtubule assembly (−fT tubulin assembly) demonstrated two distinct phases: initial assembly and then disassembly. This is consistent with the GTP cap model of microtubule dynamics, i.e., assembly took place using previously bound GTP, presumably onto GTP-capped microtubule ends, and, when this GTP was exhausted, uncapped microtubules began to disassemble. These phases were observed in buffers with and without glycerol. However, glycerol shifted the subunit/polymer equilibrium toward the polymer form during both the assembly and disassembly phases. Glycerol enhanced both the rate and extent of polymerization and then greatly slowed disassembly after the peak was reached. In fact, the concentration of polymer never returned to zero in the presence of high concentrations of glycerol, but appeared to reach an equilibrium. Centifugation at this second plateau showed an apparent critical subunit concentration of almost 1 mg/mL at 3.4 M glycerol (5 mM magnesium). Since both E-site and free GTP are absent soon after the peak of assembly is reached, this equilibrium may represent not only disassembly but also assembly of GDP subunits onto GDP polymer ends or, perhaps, isoenergetic exchange (Zeeberg & Caplow, 1981), reactions that are extremely slow or nonexistent in the absence of glycerol.

Dynamic instability, the periodic interconversion between relatively slow assembly and rapid disassembly phases of microtubule growth, is thought to be the primary mechanism of microtubule assembly both in vitro (Mitchison & Kirschner, 1984; Horio & Hotani, 1986; Walker et al., 1988) and in vivo [most recently, Sammak and Borisy (1988), Schulze and Kirschner (1988), and Cassimeris et al. (1988)]. However, evidence of these alternate phases of growth and disassembly has not been convincing in the presence of glycerol. Kristofferson et al. (1986) observed a strong inhibition of length redistribution in the presence of glycerol, indicating that disassembly phases must be either short or, possibly, absent. Similarly, preliminary experiments using video-enhanced differential interference contrast (DIC) microscopy of individual microtubules also showed an apparent suppression of the expected rapid shortening events, while elongation and nucleation rates were enhanced (E. T. O'Brien, R. W. Walker, H. P. Erickson, and E. D. Salmon, unpublished observation). However, the high rate of GTP hydrolysis at steady state in glycerol buffer (O'Brien et al., 1987) suggests that both assembly and a balancing disassembly must be taking place. The first cycle of −fT assembly also suggests that the disassembly phases characteristic of dynamic instability, although possibly of very short duration, are probably also taking place in glycerol buffer.

Although increasing concentrations of magnesium also enhanced initial microtubule assembly, it had the opposite effect on the disassembly phase of −fT assembly. Concentrations of magnesium that enhanced initial assembly also destabilized the peak of assembly, increasing the observed maximum rate of disassembly approximately 4-fold. These effects were not buffer specific; enhancement of both initial assembly and disassembly of −fT tubulin was also observed not only in glycerol but also in Pipes buffer without glycerol and in moderate concentrations of glutamate (0.1–0.5 M) (data not shown). The most straightforward explanation for these contrasting effects is that magnesium increases the net addition of GTP–tubulin onto elongating (presumably GTP capped) microtubule ends, perhaps by reducing the dissociation rate during the elongation phase [see Walker et al. (1988)]. Once GTP–tubulin has been depleted, magnesium enhances the net disassembly of GDP–tubulin from uncapped microtubules. Thus, while glycerol appears to slow the net dissociation of GDP–tubulin from GDP–tubulin polymers, in part perhaps by promoting an association of GDP–tubulin monomers onto GDP–tubulin ends, magnesium may inhibit this association or act to increase the actual dissociation rate of GDP–tubulin from disassembling microtubules. Recently we have determined, again using video-enhanced DIC microscopy, that magnesium can increase the rate of assembly during the elongation phase and also the rate of rapid disassembly after a "catastrophe" (transition from elongation to rapid shortening phase) (O'Brien et al., 1989). In Pipes buffer without glycerol (1 mM magnesium), disassembly during the rapid shortening phase is between 700 and 1000 subunits/s at 37 °C (Walker et al., 1988). Increasing magnesium concentrations increased the rate of subunit loss from each microtubule end, but appeared to affect one end more than the other. The maximum rate of subunit loss, achieved in 12.5 mM magnesium, was approximately 8000/s, while the opposite end appeared to disassemble at 2000/s.

These results are consistent with the idea that the concentration of free magnesium may play a significant role in determining the level of dynamic behavior of microtubules in the cytoplasm, and are in accord with a recent demonstration of the destabilizing effects of magnesium on microtubules in intact cells (Prescott et al., 1988). Similarly, high concen-

trations of magnesium have recently been used to produce synchronous oscillations in microtubule growth and disassembly in the presence of free GTP (Carlier et al., 1987; Pirollet et al., 1987; Mandelkow et al., 1988), a behavior consistent with our observation of an enhancement by magnesium of the rates of both net assembly of GTP–tubulin and net disassembly once GTP–tubulin had been exhausted.

At the high rate of assembly observed in glycerol, our MAP-free tubulin did not always form true microtubule, but most often formed a mixture of microtubules and long ribbons or narrow sheets. This was also the case in assembly with the analogues and ATP in the presence of glycerol. These polymeric forms have all of the important characteristics of true microtubules, except the cylindrical form. Most importantly, the tubulin subunits in the interior of a sheet are in the same bonding configuration as those in an intact microtubule. Those along an edge are indeed different, but this difference does not seem to be important biochemically. Both sheets and microtubules assemble when warmed, disassemble when cooled, hydrolyze a stoichiometric amount of GTP during assembly, and exist together in the same steady-state solution. Thus, although there is variation in the macroscopic, three-dimensional form of the polymers generated in glycerol buffer, we believe them to be equivalent to true microtubules in all essential biochemical characteristics.

Effects of the Nonhydrolyzable Analogues and ATP. The first cycle of assembly of –fT tubulin in glycerol and high magnesium provided an excellent assay for the subtle yet important effects of the nonhydrolyzable analogues and ATP on the assembly and disassembly of microtubules. The nonhydrolyzable analogues and ATP (1) enhanced microtubule assembly if E-site GTP was present, (2) stabilized assembled microtubules that had exhausted their supply of GTP, (3) did not support assembly of microtubules from pure subunits in the absence of E-site GTP, and (4) did not appear to bind to the E-site of tubulin nor to any site on free tubulin with appreciable affinity. These results have a number of interesting implications and may help clarify a number of conflicting reports in the literature.

The ability of the analogues and ATP to enhance assembly and stabilize assembled microtubules in the absence of free GTP, while not demonstrating a measurable binding affinity to the E-site of free tubulin, suggests an interaction of these nucleotides directly with the polymer. As discussed by Wyman (1964), Timasheff (Lee & Timasheff, 1977; Howard & Timasheff, 1988), and others, the presence of a ligand that binds with higher affinity to the polymeric form than to the corresponding free subunits will favor formation of the polymer, i.e., lower the critical concentration for polymerization. Similarly, a ligand that enhances assembly without binding appreciaby to free subunits must be binding preferentially to the polymer form.

The effects of ligand binding to the polymer are most easily understood in reactions that have reached an equilibrium. For a reaction as complex as microtubule assembly in the presence of GTP, the effects of differential binding to the polymer, although possibly substantial, would be difficult to assess. However, the polymerization of –fT tubulin should relax toward a true equilibrium once E-site GTP has been exhausted. To determine whether the analogues and ATP were influencing the equilibrium state or preserving a "metastable" state, we allowed first-cycle –fT tubulin to disassemble to about 50% of its peak value before adding the nucleotides. We then assessed the concentration of polymer approximately 45 min later, once an apparent equilibrium had been achieved (data not shown). As observed by turbidity, the concentration of polymer stopped decreasing once nucleotides were readded and then began to increase toward the peak level. By centrifugation of samples at 37 °C, it was confirmed that the concentration of polymer did increase substantially after addition of nucleotides almost, but not quite, to the same level achieved when the nucleotides were added before assembly had begun. This result indicates that the level of polymerization stabilized by the analogues and ATP can be reached from "below" as well as through stabilization of the assembly peak, as long as disassembly has not gone to completion. Thus, GDP–tubulin subunits that have partially disassembled from the polymerized state can reassemble in the presence of GMPPNP, GMPPCP, and ATP, and appear to reach a true equilibrium, consistent with a Wyman analysis of the effects of these nucleotides.

Preferential binding of a ligand to the polymer form can be envisioned in a number of ways. Two of the more attractive possibilities are that a conformational change in tubulin, taking place during assembly, forms a binding site not present on the free subunit or that binding sites might be formed by adjacent subunits in the microtubule lattice. In the first case, nucleotides would bind to individual subunits in assembled microtubules. In the second, nucleotides would bridge the space between adjacent subunits. The latter idea was proposed by Maccioni and Seeds (1982) to explain their observation that, in their hands, approximately 3.0 mol of nucleotide/mol of tubulin pelleted with assembled microtubules through a glycerol cushion, while only about 2 mol of nucleotide bound free, unassembled tubulin. Thus, the excess nucleotide must have been binding to a site that was present only on the assembled microtubule. They termed this binding site the polymeric, or P, site. However, although attractive in its simplicity, it is unlikely that polymeric binding sites are present uniformly down the length of each microtubule. If they were, more than one group of investigators would probably have found evidence of extra nucleotide binding to the polymer. Further, our analysis of nucleotide binding to microtubules pelleted through a sucrose cushion, or under equilibrium conditions, showed only GDP and GTP present in the polymer, with no evidence of a third, stoichiometric binding site. To explain the present data, all that is required is that the P-site exist at one or both ends of each microtubule and that binding to this site would slow the rate of subunit loss from the end. Thus, the P-site could be present at the longitudinal and lateral binding surfaces that are uniquely exposed at microtubule ends. Since the analogues and ATP stabilize the peak of assembly long after we expect GTP to be gone from the polymer, we postulate that the P-site is found on microtubule ends independent of whether or not they are capped.

Our results showing stabilization of the peak of –fT tubulin are similar to those of Carlier et al. (1988), who showed that phosphate, aluminum, and beryllium ions can stabilize the same peak of assembly. As discussed above, stabilization of the polymer by a ligand is expected if that ligand binds preferentially to the polymer. Thus, the evidence of Carlier et al. indicates only that these ions bind to a polymeric binding site, and not necessarily that this site is also the location of the γ-phosphate of GTP. Similarly, the observation that high concentrations of phosphate can competitively inhibit the binding of beryllium ions to microtubules indicates only that these ions bind to the same site, not necessarily the γ-phosphate site. There are major differences between the polymeric site proposed by Carlier et al. (1988) and that identified in the present work. Carlier et al. demonstrate stoichiometric binding of beryllium to microtubules, whereas we could not detect a

third, stoichiometric site for nucleotides. Further, our attempts to reproduce the effects of sodium phosphate on the first cycle of −fT assembly showed that, while the phosphate did stabilize a plateau when added at the assembly peak, it also strongly inhibited initial assembly when added before assembly had begun. Thus, it seems that there are two different polymeric binding sites: a stoichiometric binding site that binds ions such as beryllium and aluminum along the length of the polymer and stabilizes assembled microtubules and a substoichiometric nucleotide binding site that can also stabilize microtubules against disassembly but which also stimulates assembly. For convenience, we will refer to the nucleotide polymeric site as the P-site in the discussion that follows.

Maccioni and Seeds (1982) postulated that P-site GTP, as well as E-site GTP, might be hydrolyzed during assembly, thus supporting their earlier finding that 2 molecules of GTP are hydrolyzed for each tubulin subunit assembled into polymer (Maccioni & Seeds, 1977). However, our own work and those of many other labs are consistent with 1 GTP being hydrolyzed per tubulin monomer assembled [see O'Brien et al. (1987) for review]. Further, we saw no evidence that hydrolysis was necessary for effects mediated through the P-site. Rather, it appears that the binding of the ligand is sufficient in itself to promote assembly.

Differences between the P-Site and the Exchangeable Nucleotide Binding Site. In the present study we have been able to determine certain qualitative differences between the P- and E-sites, and some differences that, at the bulk solution level, appear only quantitative. The E-site exhibits great selectivity for GTP and GDP (Weisenberg, 1968), binds more tightly to GTP than to GDP at moderate concentrations of magnesium ion (Arai et al., 1975; Zeeberg & Caplow, 1979; Huang et al., 1985; Correia et al., 1987), and does not have an appreciable affinity for GMPPNP, GMPPCP, or ATP [consistent with some of the conclusions reached by Arai et al. (1975)]. The E-site is also a high-affinity binding site, exhibiting a k_D for GTP and GDP in the range of 10^{-6}–10^{-8} M (Arai et al., 1975; Zeeberg & Caplow, 1979). In contrast, the P-site is a relatively low affinity site, having a k_D in the range of 10^{-4} M, and appears relatively nonselective, binding guanine, adenine, and cytosine nucleotides with equal apparent affinity, as well as binding the nonhydrolyzable GTP analogues. Also, although GDP itself cannot promote assembly of tubulin under normal circumstances, the affinity of the P-site for GDP may actually be greater than that for the analogues and ATP, since low concentrations of GDP (20 μM) can reverse the stabilizing effect of 2 mM GMPPNP, GMPPCP, or ATP on the plateau of −fT tubulin assembly (data not shown).

Tubulin with GDP bound at the E-site could not assemble at the concentrations we tested, but thermodynamic arguments suggest that, at very high concentrations, GDP–tubulin should assemble. This idea is supported by observations that GDP–tubulin can, under certain conditions, assemble onto microtubule seeds (Carlier & Pantaloni, 1978). In fact, assembly without GTP hydrolysis can be very robust, as shown by assembly supported by taxol and the GDP analogues ddGDP and GMPCP (Schiff & Horwitz, 1981; Hamel et al., 1983; Sandoval et al., 1978). However, if GTP is present, hydrolysis takes place during assembly with taxol (Schiff & Horwitz, 1981; Carlier & Pantoloni, 1983), and experiments in our laboratory indicate that this hydrolysis is closely correlated with assembly (unpublished observation). Indeed, we know of no studies that convincingly demonstrate GTP present at the E-site in polymerized microtubules [see O'Brien et al. (1987)]. Thus, the evidence available indicates that, with GTP present, assembly mediated through the E-site does not take place without hydrolysis of GTP. In contrast, P-site ligands do not appear to be hydrolyzed during assembly.

Along with the large number of nucleotides that appear to interact with the P-site, it is likely that GTP can also bind. First, −fT tubulin with excess GTP added exhibited more rapid nucleation and assembly than did the same protein without GTP added (Figures 2 and 5). Since each preparation of tubulin had GTP at the E-site before assembly was started (Figure 1), the primary difference between the preparations was the presence of free GTP during assembly. Second, tubulin with 1 mM free GTP present would assemble in as little as 1.0 M glycerol, while first-cycle −fT tubulin required at least 2.5 M glycerol, with an equal concentration of tubulin present in both cases (Figure 3). It therefore seems likely that GTP promotes microtubule assembly through binding to both the E- and P-sites.

Comparison with Earlier Studies. Our finding that GMPPNP and GMPPCP did not support assembly of pure tubulin in the absence of GTP is consistent with some early studies using microtubule protein (Gaskin et al., 1974; Olmstead & Borisy, 1975) and conflicts with others (Weisenberg et al., 1976; Arai & Kaziro, 1976; Penningroth & Kirschner, 1977; Terry & Purich, 1980; Kirsch & Yarbrough, 1981). Although we cannot explain all of the positive results completely, we would suggest that most of these studies probably had not removed all of the E-site GTP. Gel filtration chromatography and dialysis removes only unbound nucleotide but leaves E-site GTP tightly bound. Charcoal, used by Penningroth and Kirschner (1977), may remove some of the E-site GTP, but is very destructive to the protein, perhaps because it may also remove N-site GTP as well as E-site (Maccioni & Seeds, 1982). Even when charcoal was used, it seems that only tubulin that still had GTP bound could assemble in the presence of GMPPNP and GMPPCP (Maccioni & Seeds, 1982). A second cycle of assembly in the presence of the analogues might have been definitive in many of these studies but was done only in the case of Gaskin et al. (1974), who also found that the analogues did not promote assembly.

An added variable in the early studies was the presence of proteins other than tubulin, which could have interfered with these studies in two general ways. First, GTP could have been regenerated from GDP by guanylate kinase activity or from ATP by nucleoside diphosphokinases. The latter activity was especially important in studies purporting to show ATP-driven microtubule assembly, as pointed out by Jacobs and Caplow (1976) and discussed further below. The second complication stems from the ability of high concentrations of MAPs to promote microtubule assembly through binding to the polymer (Sloboda et al., 1976). In the presence of MAPs and/or low concentrations of GTP, the assembly-enhancing effects of ATP and the GTP analogues might have been sufficient to support appreciable assembly.

Although the above considerations may explain some of the apparent assembly-enhancing effects of the analogues, we cannot explain the apparent inhibitory effect of GMPPCP on assembly observed by Olmsted and Borisy (1975) nor reports of binding to tubulin by radioactive GMPPNP (Arai & Kaziro, 1975) (who also reported no binding of GMPPCP or ATP to tubulin) or of incorporation of unlabeled GMPPNP into microtubules (Weisenberg et al., 1976), observations that could imply an ability to bind to the E-site of free tubulin. We observed no inhibition of assembly of −fT tubulin by any of the analogues and no indication of displacement of either GTP or GDP from the E-site by the analogues. It is conceivable

that tubulin without GTP or GDP at the E-site would demonstrate an affinity for the analogues or ATP, as demonstrated by Terry and Purich (1980). However, as evident in the work of Maccioni and Seeds (1982), removal of E-site nucleotide seems to result in denaturation of the tubulin molecule.

Although the assembly-enhancing effects of ATP were thought to be due to the replenishment of GTP via contaminating nucleoside diphosphokinase activity (Jacobs & Caplow, 1976), papers have continued to describe an independent assembly-enhancing effect of ATP (Kumagai et al., 1979; Duanmu et al., 1986). In particular, Zabrecky and Cole (1980, 1982a,b, 1983) described such an activity and concluded that it was not caused by binding to the E-site of tubulin but rather by binding to a separate site on the free tubulin subunit (1980). They then calculated the affinity of ATP for the new site as being in the range 10^{-4} M and located the ATP binding site on the α-subunit of tubulin. Our results agree with the idea that the assembly-enhancing effects of ATP are not mediated through the E-site. Further, we also estimate the binding of ATP to tubulin to be in the range 10^{-4} M. However, we believe their results are also consistent with binding of ATP to the P-site rather than to a separate site on free tubulin. Zabrecky and Cole (1980) showed that ATP would promote the assembly of microtubule ring structures. Under the conditions they used to measure ATP binding, one would expect the formation of tubulin rings or other oligomers of tubulin, which, presumably, would form P-sites. Consequently, they may have measured the affinity of ATP for the P-site. Continuing this reasoning, the photoaffinity study (Zabrecky & Cole, 1983) may not necessarily indicate the binding of ATP to free tubulin but that the adenosyl moiety of ATP is bound somewhere on the α-tubulin subunit when ATP binds to the P-site.

Assembly in GMPCPOP. Of the GTP analogues, only GMPCPOP supported assembly. This nucleotide supported multiple cycles of assembly and exhibited some interesting properties. The GMPCPOP microtubules were significantly more cold stable and could assemble at much lower concentrations of glycerol than those assembled in GTP (data not shown). This is consistent with the results of Sandoval et al. (1977), who obtained very similar results with this nucleotide. Further, the subsequent demonstration (Sandoval et al., 1978) that GMPCP also strongly promotes assembly implies, as discussed above, that GMPCPOP may be acting in part by binding to the P-site. Further study on the mechanism of assembly with GMPCP or ddGDP, in the absence of E-site GTP, should give new information as to the potential role of the P-site in microtubule assembly.

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Registry No. GTP, 86-01-1; GDP, 146-91-8; GMPPNP, 34273-04-6; GMPPCP, 13912-93-1; ATP, 56-65-5; GMPCPOP, 14997-54-7; dCTP, 2056-98-6; phosphate, 14265-44-2; L-glutamic acid, 56-86-0; glycerol, 56-81-5; magnesium, 7439-95-4; pyrophosphate, 14000-31-8.

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Mechanism of Protein-Induced Membrane Fusion: Fusion of Phospholipid Vesicles by Clathrin Associated with Its Membrane Binding and Conformational Change†

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ABSTRACT: The clathrin-induced fusion of liposome membranes, the membrane binding of clathrin, and the conformational states of clathrin were investigated over a wide pH range using large unilamellar and multilamellar vesicles composed of phosphatidylserine (PS), phosphatidylcholine (PC), PS/PC (2:1), PS/PC (1:1), or PS/PC (1:2). The pH profiles of clathrin-induced fusion of all types of liposomes containing PS showed biphasic patterns. Their pH thresholds were found in the pH range of 5–6 and shifted to lower pH values with decrease in the PS content. Similar shifts were observed in the pH profiles of clathrin binding to these vesicles, but the pH profiles of binding were different from the biphasic fusion patterns. With PC vesicles, only small degrees of fusion and clathrin binding were observed at pH 2–4. The pH dependences of the conformation and hydrophobicity of clathrin were determined by measuring the extent of the blue shift of the fluorescence maximum of 1-anilinonaphthalene-8-sulfonate in the presence of the protein, the fluorescence intensity of *N*-(1-anilinonaphthyl-4)maleimide bound to the clathrin molecule, the resonance energy transfer from its tryptophan to anilinonaphthyl residues, the partitioning of the protein in Triton X-114 solution, and the hydrophobicity index of clathrin using *cis*-parinaric acid. These measurements indicated that conformational change and exposure of hydrophobic regions occur below pH 6 and suggested that clathrin may adopt different conformational states in the pH region where it induced membrane fusion. In addition, the extents of inactivation of clathrin-induced fusion by preincubation of the protein below and above pH 4 were different. Clathrin formed insoluble aggregates at pH 4–6, and soluble aggregates below pH 4, suggesting that two distinct fusion-active states exist at pH 2–6, which might be related to the biphasic fusion patterns. These results suggest that clathrin-induced fusion of liposome membranes involves both protein binding to the membranes and a conformational change of clathrin accompanied by the exposure of its hydrophobic domains. Clathrin binding may induce close apposition of the membranes, while the conformational change may induce insertion of the protein molecule into the membrane to perturb the lipid bilayer. This study provides clues for elucidation of the general mechanisms of pH-dependent membrane fusion induced by proteins.

Membrane fusion is an important and controlled process in biological systems, such as endocytosis, exocytosis, fertilization, myoblast fusion, and intracellular transport. Recently, proteins have been recognized to participate in these membrane fusion processes. For instance, sperm–egg fusion may be mediated by a few kinds of proteins released from sperm (Monroy, 1985; Hong & Vacquier, 1986), and some glycoproteins are involved in myoblast fusion (Wakelam, 1985). In the exocytotic process, proteins are observed to form a pore to induce membrane fusion (Chandler, 1984), and exocytosis is regulated by dephosphorylation of phosphoproteins (Plattner, 1987). Synexin and synexin-like proteins are also involved in

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