ATP-induced Aggregates of Tubulin Rings*

(Received for publication, May 22, 1980, and in revised form, August 18, 1980)

James R. Zahrecky and R. David Cole
From the Department of Biochemistry, University of California, Berkeley, California 94720

Aggregates of double-walled rings (46 nm diameter) were formed upon warming (37°C) ion exchange purified bovine brain tubulin (1.5 to 2.0 mg/ml) in the presence of 1.0 mM ATP and 5.0 mM Mg²⁺. The formation of aggregated rings was blocked by GDP (1.0 mM), but when GTP (1.0 mM) was added subsequently, the block was overcome. Warming in the presence of ATP and GTP simultaneously produced a mixture of microtubules and aggregated rings. The tubulin preparations used were demonstrated to be devoid of transphosphorylation, and it is therefore clear that the action of ATP in the induction of aggregated rings was not mediated by transphosphorylation. These results are consistent with an interaction of nucleotide with tubulin at a site distinct from the previously described N- and E-sites.

A critical role is played by GTP in the assembly of microtubules (1-4). Purified tubulin noncovalently binds 2 mol of guanosine nucleotides per dimer (5). One mole is bound very tightly and is termed the nonexchangeable or N-site nucleotide; its function is still not understood. The other mole of nucleotide, bound at a position called the E-site, exchanges readily with free nucleotides. For initiation of assembly, the E-site needs to be occupied by GTP, which is subsequently hydrolyzed to GDP (3, 6, 7). Although the notion has been challenged recently (8), it was reported that GDP at the E-site will allow for the elongation of the microtubules (9).

The role of ATP is still not completely understood. The addition of ATP is effective in inducing assembly of microtubules (10, 11). ATP does not appear to bind with appreciable strength (2, 12), but there is much evidence to support a model in which ATP transphosphorylates tubulin-associated GDP, thereby promoting assembly (3, 12, 13). The transphosphorylation activity is not inherent in the tubulin but accompanies the accessory proteins resolved from tubulins by ion exchange chromatography (3, 13, 14).

It also has been suggested that ATP functions in a regulatory capacity in the assembly of tubulin into microtubules. ATP was shown to alter the assembly characteristics and stability of microtubules (15-17). Margolis and Wilson (16) demonstrated increased rates of microtubule assembly and disassembly at steady state in the presence of physiological concentrations of ATP.

As reported here, we found another effect of ATP on the polymerization of tubulin which is distinct from transphosphorylation of tubulin-bound GDP. Tubulin, prepared by ion exchange chromatography and thus devoid of transphosphorylation activity, demonstrated a reversible increase in turbidity upon warming in the presence of ATP. Examination by electron microscopy revealed that this turbidity was not due to microtubules, but to arrays of aggregated rings similar to those postulated as polymerization intermediates.

MATERIALS AND METHODS

Tubulin was prepared from fresh bovine brains using DEAE-cellulose (Whatman DE52) chromatography according to Weisenberg et al. (5), and stored at -20°C in phosphate/magnesium buffer (10 mM phosphate, 5.0 mM MgCl₂, pH 7.0) containing 1.0 M sucrose. For assembly experiments, tubulin was dialyzed overnight against approximately 400 volumes of assembly buffer (10 mM Mes, 5.0 mM MgCl₂, 1.0 mM EGTA, and 3.4 mM glycerol, pH 6.4) at 4°C. Polymerization was initiated by adding the appropriate nucleotide and then warming to 37°C in a thermostatically controlled cuvette chamber of a Cary 15 recording spectrophotometer. The increase in turbidity was monitored at 350 nm. The tubulin concentration was 1.5 to 2.0 mg/ml in all experiments.

All nucleotides were obtained from Sigma (>98% pure). These were made up as 100 mM solutions in assembly buffer without the glycerol. The nucleotide solutions were added as no more than 1% of the volume of the tubulin solution in order to minimize dilution effects.

Electron microscopy was performed in a Philips EM 301. Samples were negatively stained with 1% uranyl acetate.

RESULTS

The polymerization of tubulin induced by ATP differed from that induced by GTP. When tubulin purified by ion exchange chromatography was warmed in the presence of either 1.0 mM ATP or 1.0 mM GTP, the kinetics of polymerization were sigmoidal, as shown in Fig. 1. However, the kinetics in the presence of ATP were distinguished from those in 1.0 mM GTP by a shorter lag period, faster rate, and greater absolute turbidity increase. (The two curves in Fig. 1 were generated at the same protein concentrations.) Comparison of these samples by electron microscopy produced surprising results. Instead of the usual microtubules seen with GTP-induced assembly, the polymers induced by ATP were mostly rings aggregated in large, ordered arrays, although there were a few individual rings and occasional microtubules (Fig. 2).

These rings, which were about 46 ± 2 nm in diameter and double-walled, appeared to be similar to those described by other investigators (18, 19, 20). As with GTP-induced assembly, the turbidity increase was reversible upon cooling to 4°C. Turbidity did not develop at all when glycerol was omitted from the assembly buffer.

The induction of aggregated rings by ATP did not involve the conversion of GDP to GTP by transphosphorylation. Others have shown (3, 12, 13) that in crude preparations of microtubule proteins, ATP facilitates assembly by the enzymatic transphosphorylation of GDP to GTP. Microtubule

1 The abbreviations used are: Mes, 2-(N-morpholino)-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N',N"-tetraacetic acid; SDS, sodium dodecyl sulfate.
ATP-induced Aggregates of Tubulin Rings

Fig. 1. Polymerization of tubulin in the presence of ATP and GTP. Tubulin (approximately 1.7 mg/ml) in assembly buffer (see “Materials and Methods”) was assembled by warming to 37°C in the presence of 1.0 mM nucleotide triphosphate.

Fig. 2. Electron micrographs of polymers formed in the presence of GTP and ATP. Samples of tubulin polymerized in 1.0 mM nucleotide triphosphate and assembly buffer (see “Materials and Methods”) were negatively stained with uranyl acetate. A, polymerization in GTP (bar = 0.5 μm); B, polymerization in ATP (bar = 0.5 μm, insert bar = 0.05 μm).

Assembly occurs with the subsequent hydrolysis of the GTP. However, when tubulin that had been freed from its complement of associated proteins was warmed in the presence of 1.0 mM concentrations of ATP and GDP (combined), we found

Fig. 3. Effect of the addition of GTP on ATP-induced polymerization. Tubulin (approximately 1.5 mg/ml) was warmed (37°C) in assembly buffer and ATP (1.0 mM). At the time indicated, GTP was added to 1.0 mM, and the incubation was continued.

Fig. 4. Electron micrographs showing the effect of GTP on ATP-induced aggregates. A, sample taken when ATP-induced assembly had reached a plateau and just prior to GTP addition (see Fig. 3) (bar = 0.25 μm); B, sample taken approximately 10 min after the addition of GTP when the turbidity had reached the new, lower plateau (bar = 0.25 μm).
that there was no increase in turbidity. The failure to polymerize was not due to denaturation of the tubulin, since cooling this same sample and then adding GTP to 1.0 mM produced the usual profile of increase in turbidity when the sample was warmed to 37°C. The polymers formed in the simultaneous presence of ATP and GTP were composed of a mixture of rings and microtubules, as revealed by electron microscopy. Furthermore, the addition of GDP appeared to poison the ATP assembly reactions. This seems to confirm the absence of a transphosphorylation activity in our preparations, and we conclude that ATP exerted its effect through some other mechanism. Apparently, the transphosphorylation activity reported by others was removed effectively during the ion exchange purification.

Magnesium ions are necessary but not sufficient for the formation of aggregated rings. When tubulin was prepared in assembly buffer containing 0.1 mM MgCl₂, the addition of 1.0 mM ATP did not lead to an increase in turbidity at 37°C. The same was true at 1.0 mM MgCl₂. Although warming of tubulin in 5.0 mM Mg²⁺ gave no turbidity increase before ATP was added, the subsequent addition of ATP did result in polymerization. This demonstrated that at 1.0 mM ATP a magnesium concentration between 1.0 and 5.0 mM is necessary, and that ATP is obligatory for the assembly and aggregation of rings.

![Graph](image1.png)

**Fig. 5.** Effect of ATP on GTP-induced microtubules. Tubulin (approximately 1.7 mg/ml) was warmed (37°C) in assembly buffer and GTP (1.0 mM). ATP (to 1.0 mM) was added either early or late in GTP-induced polymerization. **A,** ATP added after 10 min; **B,** ATP added after 17 min.

![Graph](image2.png)

**Fig. 5.** Effect of ATP on GTP-induced microtubules. Tubulin (approximately 1.7 mg/ml) was warmed (37°C) in assembly buffer and GTP (1.0 mM). ATP (to 1.0 mM) was added either early or late in GTP-induced polymerization. **A,** ATP added after 10 min; **B,** ATP added after 17 min.

![Graph](image3.png)

**Fig. 5.** Effect of ATP on GTP-induced microtubules. Tubulin (approximately 1.7 mg/ml) was warmed (37°C) in assembly buffer and GTP (1.0 mM). ATP (to 1.0 mM) was added either early or late in GTP-induced polymerization. **A,** ATP added after 10 min; **B,** ATP added after 17 min.

![Graph](image4.png)

**Fig. 6.** Electron micrographs demonstrating the effect of ATP on GTP-induced microtubules. **A,** sample taken when GTP-induced assembly had just begun the elongation phase, and just prior to ATP addition (see Fig. 5A) (bar = 0.25 µm); **B,** sample taken when the turbidity recorded in Fig. 5A had reached a plateau (bar = 0.5 µm); **C,** sample taken approximately 8 min after the addition of ATP referred to in Fig. 5B (bar = 0.25 µm).

The amount of ring aggregation promoted by ATP is decreased by GTP-induced assembly of microtubules. Tubulin was warmed in the presence of 1.0 mM ATP until the turbidity had reached the plateau value. Late in the polymerization reaction, at the time indicated by the arrow in Fig. 3, GTP was added to achieve a concentration of 1.0 mM. There was an immediate drop in turbidity to a new plateau. Samples taken immediately before, and 10 min after the addition of GTP, were examined by electron microscopy. Representative micrographs are presented in Fig. 4. There appeared to be rearrangement from aggregated rings before the addition of GTP (Fig. 4A) to a mixture of rings and microtubules after the addition (Fig. 4B).
The balance between ATP-promoted ring aggregates and GTP-induced microtubules could be approached from either side. An experiment similar to the one above was performed, except that the order in which ATP and GTP were added was reversed. Tubulin was warmed in the presence of 1.0 mM GTP, then ATP was added to 1.0 mM, either early in the assembly reaction or at the plateau. When ATP was added early in the elongation phase, there was an immediate increase in the rate of turbidity formation, and the final level of turbidity also was raised (Fig. 5A). Electron microscopy (Fig. 6A, B) indicated that immediately before addition of ATP the few large polymers were all microtubules, but that after the ATP was added, there was a mixture of rings and microtubules. When ATP was added after the GTP assembly reached steady state, there was initially a small, sudden increase in turbidity which was followed by a slower, steady rise (Fig. 5B). We interpret the sudden increase as representing the formation of rings by the interaction of ATP with the unpolymerized tubulin that had been in equilibrium with microtubules. Then, as the microtubule-tubulin equilibrium shifted toward disassembly, more rings were formed at a rate limited by the speed with which free tubulin became available. Electron microscopy revealed that the addition of ATP caused a transition from polymers exclusively in the form of microtubules to a mixture of rings and microtubules (Fig. 6C).

**DISCUSSION**

The assembly properties of tubulin purified by ion exchange chromatography are dissimilar in the presence of ATP and GTP. ATP leads to the formation of microtubules that are morphologically like those observed in vitro, while ATP induces the formation of 46 nm diameter, double-walled rings that have the ability to aggregate into large, two-dimensional arrays. Comparable rings have been seen in several laboratories, and they have been postulated to function as intermediates in the pathway of microtubule assembly (21, 22). Electron micrographs have been presented (21) which seemed to show double-walled rings adding to the ends of microtubules. Similar images were occasionally seen among the arrays of aggregated rings induced by ATP.

The only previous report of extensively aggregated rings was that of Voter and Erickson (20). Although these workers found some radially triple- and quadruple-walled rings the aggregates they reported contained double-walled rings with an outer diameter of 46 nm and an inner diameter of 24 nm. We found exclusively double-walled rings with just those dimensions. Moreover, as in the previous case, the aggregates we observed exhibited two binding modes, one in which rings overlapped at their junction and another in which rings made side-by-side contact without substantial overlap. Although the arrangement of these two modes of binding was somewhat irregular, the most common arrangement was one in which any particular ring had a pair of nonoverlapping contacts, diametrically opposed, and four other contacts of the overlapping type which gave the overall array of rings the appearance of a tetragonal lattice. Despite the two-dimensional appearance of the lattice, the overlapping of rings reveals that there is aggregation in the third dimension. However, since there is no further aggregation in that dimension, it is implied that each ring has only one face capable of aggregation by overlap. In any case, the aggregated rings in the present study were identical in all particulars to those of Voter and Erickson.

Surprisingly, while the structure reported by Voter and Erickson was just like ours, the conditions under which it formed were very different. The aggregated rings described by these investigators were formed in the absence of glycerol and were found to require magnesium concentrations 3 to 9 times higher than ours. In contrast, our aggregated rings were formed in the presence of glycerol under conditions that normally lead to microtubule formation, except that ATP was substituted for GTP in the assembly buffer. One consequence of the difference in conditions is that Voter and Erickson found it necessary to lower the concentration of divalent cations in order to disassemble their aggregates, while in our case, simply reducing the temperature was sufficient.

The reason other workers have not observed the induction of aggregated rings by ATP is not clear, but it ought to be kept in mind that their preparations contained non-tubulin proteins that were removed from our preparations. Most studies of microtubule assembly have been carried out on microtubule proteins purified by repeated cycles of assembly and disassembly. Tubulin prepared in this manner is only 75 to 85% pure, as indicated by SDS-gel electrophoresis (18, 23). The non-tubulin proteins that co-purify with tubulin are thought to be involved in assembly (23–26), although they are not obligatory since tubulin prepared by methods that remove these associated proteins can also assemble into microtubules (27, 28). An enzyme that is associated with preparations of accessory proteins is a transphosphorylase that converts tubulin-bound GDP to GTP. Tubulin prepared by cycles of assembly-disassembly can be polymerized into microtubules by the addition of ATP and GTP (combined) at levels comparable to those used in our experiments (12). Furthermore, others demonstrated that along with the fraction containing accessory proteins, the transphosphorylase activity could be removed from tubulin by chromatography on phosphocellulose (3) or DEAE cellulose (13). Jacobs (14) separated the transphosphorylase and then showed that the ATP, GDP assembly system could be reconstituted with the enzyme. Since our tubulin, prepared by DEAE cellulose chromatography, failed to polymerize when ATP and GDP were added, we conclude that our preparations were devoid of transphosphorylase, and, presumably, most other nontubulin components of the microtubule system.

It is clear that, in addition to its role in transphosphorylation of GDP to GTP at the E-site of tubulin, ATP affects tubulin directly. The interaction of ATP with tubulin seems to be at a site distinct from the previously described N- and E-sites that bind GTP. ATP and GTP were each capable of producing their own distinct effects, even when one nucleotide was added after the reaction with the other had come to equilibrium. When 1.0 mM GDP was present at the same time as ATP there was no polymer formation; however, the subsequent addition of GTP caused the formation of a mixture of rings and microtubules. The inhibition of polymerization by GDP can be understood by the suggestions of previous workers that the presence of GDP in the E-site does not allow nucleation (7, 9). The addition of GTP to the GDP-tubulin system produces polymers, presumably with the replacement of GDP by GTP at the E-site or by transphosphorylation. Similarly, in the present experiments, GTP overcame the inhibition by GDP and allowed ATP to induce the formation of aggregated rings. Apparently, the E-site must be in the GTP form for ATP to produce the rings. This implies that ATP acts at a place distinct from the E-site. Since the release of GTP from the N-site has not been reported under normal conditions, it is reasonable to assume that GTP was not displaced by ATP, and that the ATP must have acted elsewhere. Other workers have proposed a third site on tubulin for nucleotide interaction to explain the effects of high nucleotide concentrations on assembly and disassembly (8) and as part of the transphosphorylation mechanism (2). However, the relevance of these reports to the site involved in aggregated ring formation is an open question at this time. Our future experiments will be
ATP-induced Aggregates of Tubulin Rings

designed to test the idea of a third nucleotide interaction site and, if it exists, to elucidate its mode of action in the assembly of aggregated rings induced by ATP.

Acknowledgments—We are grateful to Alice Taylor for assistance in electron microscopy.

REFERENCES
1. Weisenberg, R. C. (1972) Science 177, 1104-1105
2. Jacobs, M., Smith, H., and Taylor, E. W. (1974) J. Mol. Biol. 89, 450-468
3. Penningroth, S. M., and Kirschner, M. W. (1977) J. Mol. Biol. 115, 643-673
4. Weisenberg, R. C., Deery, W. J., and Dickinson, P. J. (1976) Biochemistry 15, 4248-4254
5. Weisenberg, R. C., Borsay, G. G., and Taylor, E. W. (1968) Biochemistry 7, 4466-4479
6. Kobayashi, T. (1975) J. Biochem. (Tokyo) 77, 1193-1197
7. David-Pfuty, T., Erickson, H. P., and Pantaloni, D. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5372-5376
8. Jameson, L., and Caplow, M. (1980) J. Biol. Chem. 255, 2284-2292
9. Carlier, M., and Pantaloni, D. (1978) Biochemistry 17, 1908-1915
10. Olmsted, J. B., and Borsay, G. G. (1973) Biochemistry 12, 4282-4289
11. Borsay, G. G., Olmsted, J. B., Marcum, J. M., and Allen, C. (1974) Fed. Proc. 33, 167-174
12. Jacobs, M., and Caplow, M. (1976) Biochem. Biophys. Res. Commun. 68, 127-135
13. Zeeberg, B., and Caplow, M. (1978) J. Biol. Chem. 253, 1984-1990
14. Jacobs, M. (1975) Ann. N.Y. Acad. Sci. 253, 562-572
15. Selkoe, D. J. (1979) Brain Res. 172, 382-386
16. Margolis, R. L., and Wilson, L. (1975) Cell 18, 673-679
17. Kumagai, H., Nishida, E., and Sakai, H. (1979) J. Biochem. (Tokyo) 85, 495-502
18. Kirschner, M. W. (1978) Int. Rev. Cyt. 54, 1-71
19. Timasheff, S. N., Frigon, R. P., and Lee, J. C. (1976) Fed. Proc. 35, 1886-1891
20. Voter, W. A., and Erickson, H. P. (1979) J. Supramol. Struct. 10, 419-431
21. Erickson, H. F. (1974) J. Supramol. Struct. 2, 393-411
22. Kirschner, M. W., Honig, L. S., and Williams, R. C. (1975) J. Mol. Biol. 99, 263-276
23. Murphy, D. B., and Borsay, G. G. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2696-2700
24. Borsay, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B., and Johnson, K. A. (1975) Ann. N.Y. Acad. Sci. 253, 107-132
25. Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., and Kirschner, M. W. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1858-1862
26. Sloboda, R. D., Dentler, W. L., and Rosenbaum, J. L. (1976) Biochemistry 15, 4497-4503
27. Lee, J. C., and Timasheff, S. N. (1975) Biochemistry 14, 5183-5187
28. Lee, J. C., Hirsh, J., and Timasheff, S. N. (1975) Arch. Biochem. Biophys. 168, 726-729