PROPERTIES OF TUBULIN TREATED WITH ALKALINE PHOSPHATASE TO REMOVE GUANINE NUCLEOTIDES FROM THE EXCHANGEABLE BINDING SITE

Daniel L. PURICH and Robert K. MacNEAL
Department of Chemistry, University of California, Santa Barbara, CA 93106, USA

Received 22 September 1978

1. Introduction

Tubulin is the principal protein component of microtubules, and there has been much interest in the interactions of this protein with guanine nucleotides. The heterodimeric structure of tubulin is apparently reflected in the asymmetric nature of the guanine nucleotide sites: one is exchangeable with added nucleotide and the other is nonexchangeable [1,2]. Recently, we demonstrated that GTP-supported assembly of microtubules is attended by a stoichiometric hydrolysis of the exchangeable-site GTP but not the nonexchangeable GTP [3]. It has also been possible to demonstrate that the microtubule-associated proteins (MAPs) play a role in stimulating both assembly and GTP hydrolysis at the exchangeable site [3,4]. Nonetheless, there are a number of intriguing aspects to this process, and the observation [5–7] that nonhydrolyzable β–γ analogues, such as GMP-P(CH_2)P and GMP-P(NH)P, can support assembly has been especially provocative. To initiate assembly with these analogues, however, one must first remove exchangeable nucleotide by laborious protocols involving charcoal treatment or exchange replacement by GMP–P(CH_2)P followed by gel filtration [7–9], and the yield of assembly-competent tubulin is low [7,9]. In addition, as much as 40% of the exchangeable nucleotide remains even after such treatment [9]. Another laboratory has reported isolation of a tubulin·colchicine complex with empty exchangeable sites (3-sites) [10]. However, such tubulin is assembly-incompetent. For these reasons, we were interested in a more suitable protocol. It was reasoned that one might take advantage of the resistance of GMP–P(CH_2)P, but not GTP or GDP, to alkaline phosphatase and to use a brief exposure to alkaline phosphatase to destroy the exchangeable nucleotides. A description of this new approach and several important properties of the microtubule reassembly process form the basis for this report.

2. Experimental procedures

2.1. *Materials*

GTP, GDP, GMP–P(CH_2)P, L-glutamic acid, and calf intestinal mucosa alkaline phosphatase (1000 units/mg) were Sigma products. [8-^{3}H]GDP was purchased from Schwarz-Mann.

2.2. *Methods*

Beef brain tubulin was prepared by 2 cycles of assembly/disassembly as in [3] and stored at –80°C. The buffer used contained 100 mM glutamic acid, 20 mM MES, 1 mM $MgCl_2$, and 1 mM EGTA, adjusted to pH 6.8 with KOH. EGTA was excluded during the final purification (see fig.1 legend). Assembly at 37°C was monitored at 350 nm on a Cary 118 spectrophotometer. Rates of assembly were determined by drawing a tangent to the assembly curve at the steepest point. Percent assembly of tubulin into microtubules (w/w) was determined from a standard curve relating light scattering at 350 nm to weight concentration of microtubules [3]. Liquid scintillation counting was done with a xylene-base fluor using a Beckman LS 3155T scintillation counter. Protein was determined by the method in [11].

3. Results

The tight binding of GDP and GTP represents a major problem for those characterizing tubulin–nucleotide interactions; gel filtration and dialysis are practically ineffective in resolving the nucleotide from the binding site on tubulin [3]. We have determined that brief exposure of microtubular protein to calf intestinal mucosa alkaline phosphatase can remove the nucleotide by hydrolysis. As shown in fig.1A, there is essentially no microtubule assembly in 1.0 mM GMP–P(CH_2)P when the bound exchangeable GDP is not removed. On the other hand, tubulin exposed to alkaline phosphatase (3 units/mg tubulin) and GMP–P(CH_2)P (1.0 mM) polymerized after a 2–3 min lag. The lag in assembly and the rate of microtubule assembly depends upon the amount of added alkaline phosphatase. This is demonstrated in fig.1B. A considerable amount of enzyme is necessary to obviate the apparent poor affinity of the phosphatase for nucleotides.

The presence of numerous microtubules was confirmed by negative-stain electron microscopy. These microtubules are resistant to Ca^{2+}-induced depolymerization as judged by light scattering and electron microscopy. GMP–P(CH_2)P-induced microtubules exhibit normal sensitivity to cold temperatures. After cold depolymerization, microtubules will reassemble upon warming to 37°C. This agrees with the results in [7]. The extent of microtubule assembly seen in fig.1 is 73% (w/w) after 40 min. Another report has indicated that Zn^{2+} can induce formation of sheet structures [12]. However, nearly all of the increase in light scattering seen in fig.1 must be due to the action of alkaline phosphatase.

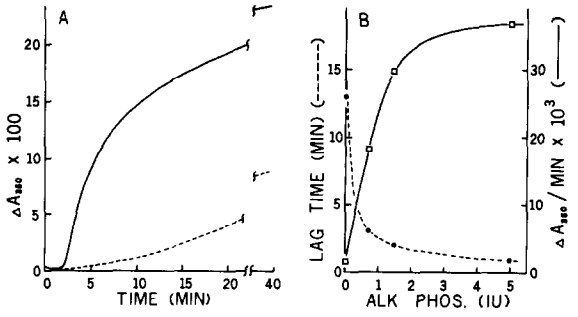

Fig.1. Polymerization of tubulin at 37°C with or without alkaline phosphatase. Tubulin (1.4 mg/ml) was purified by assembly using 0.1 mM acetyl phosphate and 0.1 unit acetate kinase/mg tubulin. Following warm centrifugation, the pellet was resuspended in EGTA-free buffer. After cold disassembly the tubulin was centrifuged at 45 000 × *g* for 25 min to remove insoluble aggregates. The supernatant was then gel filtered on a 1.5 × 28 cm Sephadex G-25 column at 2°C to remove traces of EGTA. GMP–P(CH_2)P (1 mM), $ZnCl_2$ (0.03 mM) and alkaline phosphatase (0–5 units) were added at the start of the assay. Volume was 0.9 ml. (A) Assembly as a function of time: upper curve, 5.0 units alkaline phosphatase; lower curve, no alkaline phosphatase. (B) Lag time and rate of assembly as a function of alkaline phosphatase added: solid line, rate of assembly, defined as described in the text; dashed line, lag time before onset of assembly.

To further demonstrate that hydrolysis of guanine nucleotide is responsible for GMP–P(CH_2)P-induced assembly, we carried out an analysis of nucleotide content at various intervals after phosphatase addition. For this purpose, a trace amount of [8-^{3}H]GDP was exchanged into the E-site by preincubation at 10°C for 150 min. After enzyme addition, samples were withdrawn at specified intervals and quenched by addition of 0.4 vol. 20 mM EDTA to sequester Zn^{2+} necessary for phosphatase activity [13]. The tubulin samples were immediately spotted on a DEAE cellulose thin-layer plate and taken to dryness. As shown in fig.2, the E-site nucleotide is rapidly degraded, and the lag in assembly corresponds to the time necessary to reduce the bound GDP to ~5% of its original level. It is noteworthy that this procedure results in 97% release of guanine nucleotide, a value superior to the 60% value obtained by earlier methods [7,9]. Also of interest is the observation that the hydrolysis rate is consistent with the half-life for GDP release from the tubulin·GDP complex at 37°C (B. J. Terry and D. L. P., unpublished findings). This suggests that the phosphatase acts on free and not bound nucleotide.

As further indication that the exchangeable nucleotides may be rapidly evacuated to extents in excess of earlier work, we measured the rate of polymerization at various GMP–P(CH_2)P levels. The findings of such an experiments are presented in fig.3. A value of 0.12 mM corresponds to the concentration yielding half-maximal stimulation of assembly by the phosphonate analogue of GTP. Earlier studies [9] indicated a corresponding value of about 0.5 mM, presumably due to the inhibitory action of tubulin exchangeable-site·GDP complex. GDP is a potent inhibitor of GTP- and Cr(III)GTP-induced assembly

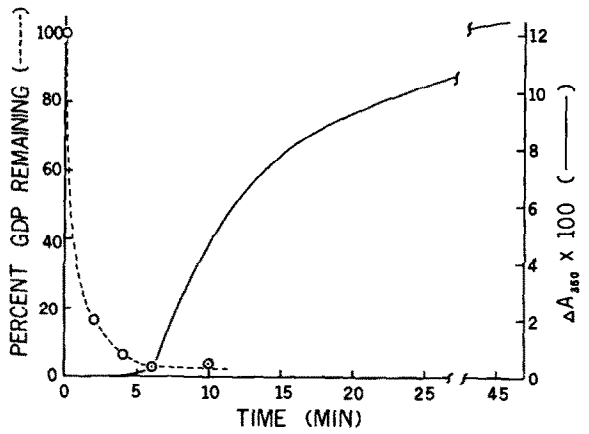

Fig.2. Rate of hydrolysis of exchangeable GDP and microtubule assembly in the presence of alkaline phosphatase and GMP–P(CH_2)P. Tubulin (1.1 mg/ml) was purified by assembly/disassembly and cold centrifugation as in fig.1 legend. A trace amount of [8-^{3}H]GDP was added and allowed to equilibrate with endogenous E-site GDP at 10°C for 150 min. The mixture was then gel filtered as in fig.1 legend. Exchangeable GDP was not removed. GMP–P(CH_2)P (0.56 mM), $ZnCl_2$ (0.03 mM), and alkaline phosphates (4.0 units) were then added. Volume was 1.1 ml. A 25 μl aliquot was withdrawn and mixed with 10 μl 20 mM EDTA. An aliquot (7 μl) of this mix was then spotted within 2 min on a DEAE cellulose thin-layer chromatography plate. The tubulin mix was then warmed to 37°C and 25 μl aliquots removed at 2, 4, 6 and 10 min. The aliquots were quenched and spotted as described above. The spots were developed using 32 mM HCl and located with a marker mix of GTP, GDP and GMP. Each spot was counted in a xylene-base fluor. The reaction product comigrated with GMP. Dashed line, percent GDP remaining as a function of time, determined as the ratio of cpm [8-^{3}H]GDP to total cpm; solid line assembly monitored by light scattering.

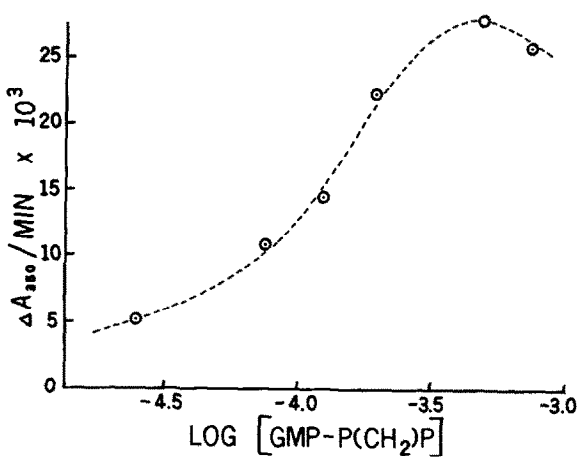

Fig.3. Dependence of the rate of microtubule assembly on the concentration of GMP–P(CH_2)P. Tubulin (1.5 mg/ml) was purified as described in the legend to fig.1 except that the cold centrifugation step was omitted. The assembly mixture contained alkaline phosphatase (4.0 units), $ZnCl_2$ (0.03 mM) and GMP–P(CH_2)P (0.025–0.750 mM). Volume was 0.9 ml.

[3,14], as well as GMP-P(CH_2)P [9]. Thus, our value of 0.12 mM for half-maximal stimulation of assembly rate is consonant with the observed efficient hydrolysis of guanine nucleotides by the phosphatase.

4. Discussion

The use of alkaline phosphatase to degraded exchangeable GDP-associated with tubulin has proven to be simple and practical. Further simplication is possible. For example, the need for gel filtration could be eliminated by centrifugation of microtubules through a sucrose cushion. Alternately, EGTA could be excluded from the second purification cycle. Preincubation of tubulin·GDP with alkaline phosphatase at 0–10°C may enhance the polymerization properties even further.

In addition, it should prove possible to isolate tubulin with vacant E-sites by an extension of the method presented. Immobilized alkaline phosphatase is commercially available (Sigma). One should be able to pass tubulin solutions over a column of this material and obtain tubulin with vacant E-sites in the effluent. A number of interesting experiments could be conducted using such tubulin. For example, the nonexchangeable guanine nucleotide may be unaffected by this treatment. By removing the exchangeable nucleotide, study of the nonexchangeable site would be greatly facilitated.

The methods presented here should permit easy and inexpensive isolation of bulk quantities of tubulin with GMP–P(CH_2)P at the E-site. This would permit investigation of a number of questions relating to the role of GTP hydrolysis. For example, GTP hydrolysis has been proposed to permit microtubule depolymerization at a later time [15]. Using the protocol in [16], it should prove possible to investigate their

proposal. Other questions relating to the interaction of anti-mitotic drugs and GMP–P(CH_2)P-induced microtubules could be investigated systematically.

Acknowledgements

This research was supported in part by an appropriation from the University of California Cancer Research Coordinating Committee. D. L. P. is the recipient of a NIH Research Career Development Award and an Alfred P. Sloan Fellowship. We thank Dr Charles Y. Huang for a helpful discussion about alkaline phosphatase action.

References

[1] Weisenberg, R. C., Borisy, G. G. and Taylor, E. W. (1968) Biochemistry 7, 4466–4479.
[2] Berry, R. W. and Shelanski, M. C. (1972) J. Mol. Biol. 71, 71–80.
[3] MacNeal, R. K. and Purich, D. L. (1978) J. Biol. Chem. 253, in press.
[4] Sloboda, R. D., Dentler, W. L. and Rosenbaum, J. L. (1976) Biochemistry 15, 4497–4505.
[5] Weisenberg, R. C., Deery, W. J and Dickinson, O. J. (1976) Biochemistry 15, 4248–4254.
[6] Arai, T. and Kaziro, Y. (1976) Biochem. Biophys. Res. Commun. 69, 369–376.
[7] Penningroth, S. M, Cleveland, D. W. and Kirschner, M. W. (1976) Cold Spring Harbor Symp. Cell Proliferation (Goldman, R. et al. eds) vol. III, pp. 1233–1257.
[8] Penningroth, S. M. and Kirschner, M. W. (1977) J. Mol. Biol. 115, 643–673.
[9] Penningroth, S. M. and Kirschner, M. W. (1978) Biochemistry 17, 734–740.
[10] Arai, T., Ihara, K. and Kaziro, Y. (1975) J. Biochem. (Tokyo) 77, 647–658.
[11] Lowry, O. H., Rosebrough, N. J , Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[12] Gaskin, F. and Kress, Y. (1977) J. Biol Chem. 252, 6918–6924.
[13] Engstrom, L. (1961) Biochim. Biophys. Acta 52, 36.
[14] MacNeal, R. K. and Purich, D. L. (1978) Arch. Biochem. Biophys. in press.
[15] Weisenberg, R. C. and Deery, W. J. (1976) Nature 263, 792.
[16] Margolis, R. and Wilson, L. (1978) Cell 13, 1–8.