NEUROTUBULIN POLYMERIZATION AND PHOSPHORYLATION REACTIONS CATALYZED BY 'ASSOCIATED' PROTEIN KINASE

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1. Introduction

Procedures recently described [1–3] allow purification of neurotubulin which was able to polymerize, producing reconstitued microtubules. It therefore became possible to reinvestigate several problems related to the presence of cAMP dependent protein kinase [4–8], which is currently co-purified with tubulin and to verify, if during the polymerization process tubulin serves as a substrate [4–7] for this enzyme.

2. Materials and methods

2.1. *Purification of rat brain tubulin*

Tubulin was prepared by the aggregation–disaggregation method of Shelanski [2], slightly simpliflied as follows: the supernatant (1 hr, 105 000 *g*) of an homogenate (v/w) prepared in buffer A (MES 0.1 M; pH 6.4; EGTA 1mM; GTP 1 mM; $MgCl_2$ 0.5mM) was mixed with the same volume of 8 M glycerol in MES buffer (buffer B).

After 30 min incubation at 37°C, the sample was layered on 7 ml 6 M glycerol in MES buffer (38 ml centrifuge tubes) and centrifuged at 24°C for 1.5 hr at 35 000 rpm (rotor R 50-1). The supernatant was removed by aspiration and the walls of the tubes were dried with filter paper before dissociation of the microtubules in buffer A at 4°C for 30 min. The dissociated preparation (hand homogeneized with a Teflon-pestle potter) was centrifuged for 1 hr at 105 000 *g* at 4°C. The final supernatant contained 80–90% pure tubulin which exhibited nearly the same polyacrylamide gel profile as tubulin prepared according to a 3 cycles assembly–disassembly purification of Gaskin et al. [9].

2.2. *Polymerization and phosphorylation of microtubules*

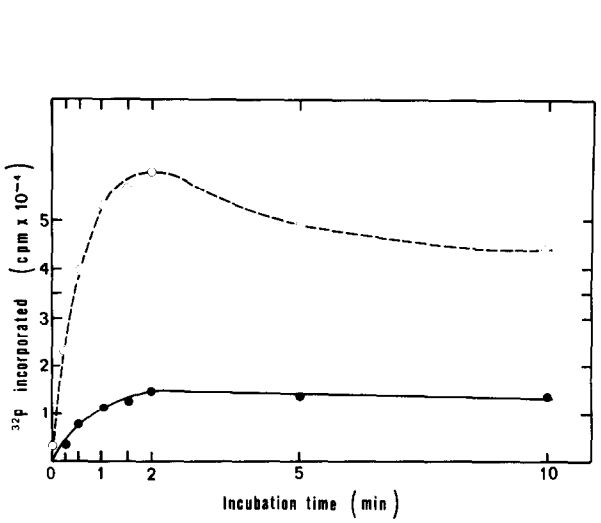
These reactions were performed under the same conditions. Tubulin was in buffer A and the phosphorylation assay contained 2×10^{-5} M γ-^{32}P-ATP and 5×10^{-6} M cAMP when added. Protein bound ^{32}P was measured as described by Reimann et al. [10]. Polymerization was followed as described by Shelanski et al. [2]. Buffer A and not 4 M glycerol was used in order to allow a further rapid depolymerization of the microtubules by incubation at 4°C for 30 min since microtubules polymerized in the presence of 4 M glycerol cannot depolymerize in this medium [2].

2.3. *Sucrose gradient ultracentrifugation*

Linear gradients (5–20%; 50–70%) were prepared with sucrose solutions in buffer A. Ultracentrifugations were performed in a SW 50L rotor at 50 000 rpm. 50–70% sucrose gradients were run 45 min at 24°C for the polymerized microtubules and at 4°C for the cold-depolymerized preparation. 5–20% sucrose gradients of cold-depolymerized tubulin were run 14 hr at 50 000 rpm and 4°C.

2.4. *Polyacrylamide gel electrophoresis*

The samples were reduced and alkylated iodoacetamide using the method of Renaud et al. [11]. Electrophoresis with 1% SDS was performed as described by Eipper [12].



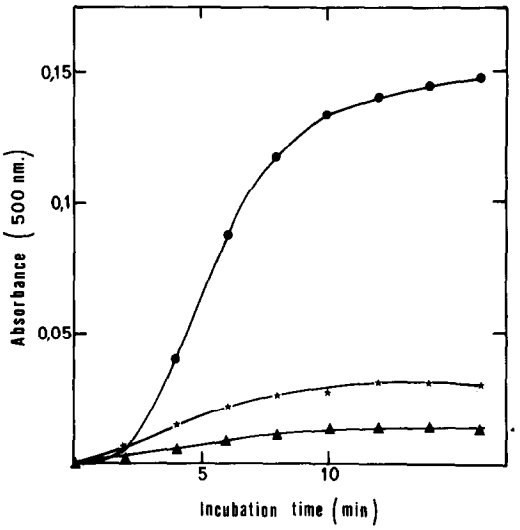


Fig. 1A. Kinetics of auto-phosphorylation of a purified tubulin preparation performed in buffer A containing 5 X 10^{-6} M cAMP and 2 X 10^{-5} M γ-^{32}P-ATP. Tubulin concentration was 5 mg/ml. The incubation was done at 37°C. Aliquots of 100 μl were layered on discs of Whatmann 3 MM paper previously soaked in 10% TCA; discs were washed 3 times with TCA, once with alcohol, once with ether and dried; radioactivity was counted by Cerenkov effect. (o—o) plus cAMP; (•---•) minus cAMP
Fig. 1B. Kinetics of polymerization of a purified tubulin (5 mg/ml) preparation incubated in buffer A at 37°C in a thermostated Gilford spectrophotometer. Absorbance was recorded at 500 nm. No addition (•---•); colchicine 10^{-4} M (★---★); Ca^{2+} 2.0 mM (▲---▲).

3. Results

Tubulin, purified by the aggregation–disaggregation method of Shelanski [2], was allowed to polymerize in the presence of γ-^{32}P-ATP. Phosphorylation (fig. 1a) and polymerization (fig. 1b) were followed parallel in the presence and in the absence of cAMP. cAMP had no significant effect on polymerization. Fig. 1a also shows that under the conditions of polymerization (fig. 1b), the tubulin preparation was phosphorylated and that this reaction was cAMP dependent. At the end of the polymerization, the phosphorylated material was pelleted by ultracentrifugation at 105 000 *g*, 1 hr, through a layer of buffer B. The pellet was dissolved at 4°C in buffer A and then reduced and alkylated as described in Materials and methods. The solution obtained was studied by polyacrylamide gel electrophoresis. Fig. 2 shows that ^{32}P does not migrate with the tubulin chains but at the level of the minor components which are always co-purified with tubulin when prepared by the method of Gaskin et al. [9].

The pellet obtained during the polymerization–

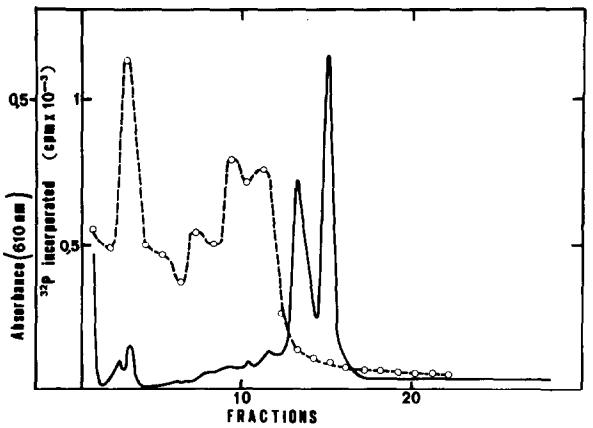


Fig. 2. 7.5% polyacrylamide gel electrophoresis of a repolymerized phosphorylated preparation of microtubules. Microtubules were isolated by centrifugation (105 000 *g* for 1 hr at 4°C) through a layer of buffer B, dissociated at 4°C during 30 min. and dialyzed against 8M urea, 0.12 M MSH. The reduced preparation was alkylated by iodoacetamide. The densitometer tracing (——) of the stained gel (Amido Black) was performed using a Gilford scanning densitometer. Gel was sliced into 1 mm fractions and the radioactivity counted (o---o).

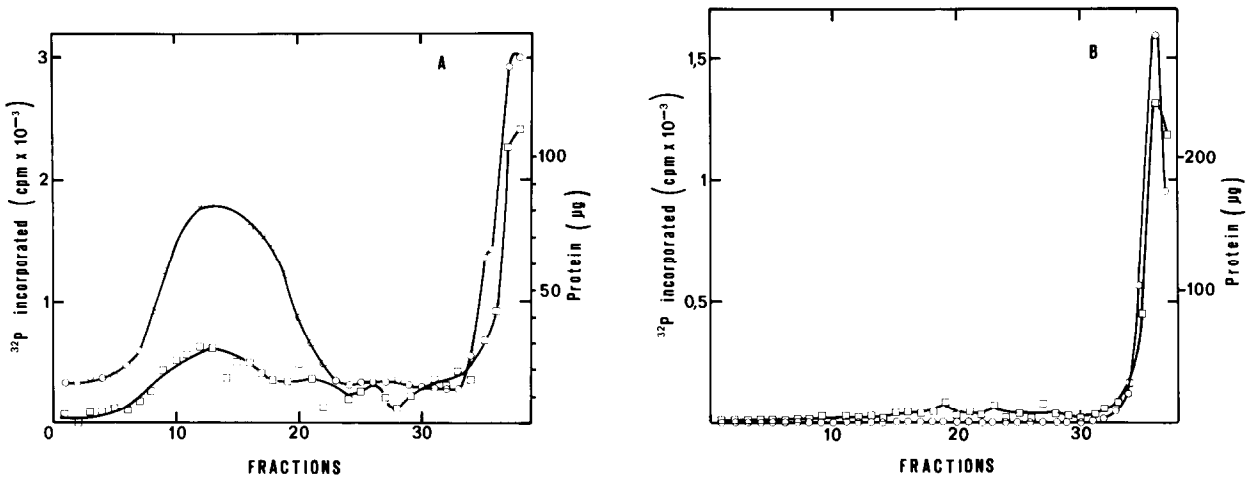


Fig. 3. Ultracentrifugation on sucrose gradients (50–70%) of a microtubule preparation polymerized and phosphorylated in conditions of fig. 1. Centrifugation was performed in Spinco SW 50-1 rotor at 50 000 rpm for 45 min. The gradients were collected and aliquots of each fraction were used for protein determination (□——□) and TCA precipitable radioactivity (○---○). A) Centrifugation was performed at 24°C immediately after polymerization. B) The incubate was maintained at 4°C for 30 min in order to dissociate the microtubules and then layered on a sucrose gradient which was run at 4°C.

phosphorylation experiment was also directly studied by ultracentrifugation on a very dense gradient (50–70%). Under these conditions, ^{32}P sediments (fig. 3a) with very heavy structures (several hundred S), probably reconstituted microtubules [1, 2]. However, if the pellet was maintained at 4°C during 30 min before sucrose gradient ultracentrifugation, the peak of very heavy labelled material disappeared (fig. 3b); at this temperature, the microtubules dissociate to the 6 S subunit [1]. Further ultracentrifugal analysis of this material was performed using a 5–20% sucrose gradient (fig. 4); under these conditions, ^{32}P sedimented at the level of the 6 S peak; however the highest ^{32}P/protein ratio was found at the level of material heavier than 6 S. Moreover tubulin 6 S was surely not phosphorylated as demonstrated by the polyacrylamide gel electrophoresis profile (fig. 2). Therefore ^{32}P found at the 6 S level in fig. 4 likely corresponds to non-tubulin molecules which are separated by this technique.

4. Discussion

The results reported in this paper show that tubulin obtained by the Shelanski procedure contains an associated cAMP dependent protein kinase which does not phosphorylate the tubulin itself but the minor constituents present in the preparation. These constituents co-polymerize with tubulin producing very heavy material which is likely formed by reconstituted microtubules.

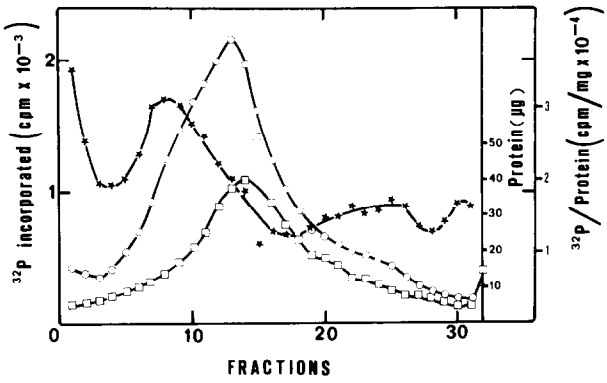


Fig. 4. Ultracentrifugation on a sucrose gradient (5–20%) of a microtobule preparation, polymerized and phosphorylated in conditions of fig. 1 and then depolymerized at 4°C for 30 min. The dissociated tubulin was visualized by protein determination (□——□); ^{32}P TCA precipitable radioactivity (○——○) was determined and ^{32}P/protein ratio (★——★) was calculated.

These ^{32}P-labelled constituents have a much lower sedimentation coefficient after exposure at 4°C of the polymerized labelled material. Similar results were obtained using colchicine (10^{-4}M) [13] or Ca^{2+} (2×10^{-3}M) (unpublished results), agents which inhibit the polymerization of tubulin [1,2].

These results clearly show that then prepared by the Shelanski procedure, tubulin is not a substrate for the associated cAMP dependent protein kinase. Contrarily, as previously reported [5, 6] when tubulin was prepared by the Weisenberg batch procedure the aggregates were actually labelled in vitro by ^{32}P. However, this tubulin preparation cannot polymerize in structures larger than 40 S. This is in contrast to the method of Shelanski et al. which gives tubulin capable of polymerizing to large microtubules as shown by electron microscopy [2]. It is therefore possible that some denaturation of tubulin is produced during the batch procedure yielding a molecule which is unable to polymerize but able to be phosphorylated.

The nature of the minor constituents which are actually phosphorylated in vitro is unknown. However, brain tubulin preparations contain a dynein-like molecule [9, 4], ATPases [9], transphosphorylase [15], triphosphatase [15] and protein kinase [4–7] activities, as well as other unknown molecules with high molecular weights [9]. The fact that minor constituents are phosphorylated and that they co-polymerize with tubulin suggests either that they participate in the structure (and/or the function) of the microtubule organelle or that they are artefactually absorbed by the microtubules. Further studies are necessary in order to answer these important questions.

Acknowledgements

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References

[1] Weisenberg, R. C. (1972) Science 177, 1104–1105.
[2] Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 765–768.
[3] Borisy, G. G. and Olmsted, J. B. (1972) Science 177, 1196–1197.
[4] Goodman, D. B. P., Rasmussen, H., Dibella, F. and Guthrow, C. E. (1970) Proc. Natl. Acad. Sci. U. S. 67, 652–659.
[5] Rappaport, L., Leterrier, J. F. and Nunez, J. (1972) FEBS Letters 26, 349–352.
[6] Leterrier, J. F., Rappaport, L. and Nunez, J. (1974) Molecular and Cellular Endocrinology, 1, 65–75.
[7] Eipper, B. A. (1974) J. Biol. Chem. 249, 1398–1406.
[8] Soifer, D., Laszlo, A. H. and Scotto, J. M. (1972) Biochim. Biophys. Acta, 271, 182–192.
[9] Gaskin, F., Kramer, S. B., Cantor, C. R., Velstein, R. A. and Shelanski, M. L. (1973) FEBS Letters, 40, 281–286.
[10] Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1986–1995.
[11] Renaud, F. L., Rowe, A. J. and Gibbons, I. R. (1968) J. Cell Biol. 36, 79–90.
[12] Eipper, B. A. (1972) Proc. Natl. Acad. Sci. U. S. 69, 2283–2287.
[13] Rappaport, L., Leterrier, J. F. and Nunez, J. (1974) Conference on the biology of cytoplasmic microtubules, New York Acad. Sci. (in press)
[14] Burns, R. G. and Pollard, T. D. (1974) FEBS Letters 40, 274–280.
[15] Jacobs, M. and Taylor, E. (1974) Conference on the biology of cytoplasmic microtubules. New York Acad. Sci. (in press).