RESOLUTION OF CYCLIC AMP STIMULATED PROTEIN KINASE FROM POLYMERIZATION-PURIFIED BRAIN MICROTUBULES.*

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Polymerization-deploymerization purified microtubules from mouse brain contain, in addition to tubulin, several minor proteins, including protein kinase activity. The protein kinase copurifies with microtubules in constant proportion to tubulin through two, three, or four cycles of polymerization; it can be resolved from tubulin by gel filtration chromatography and has an apparent molecular weight of 280,000. Its activity is stimulated 7-fold by cyclic AMP, and resembles the soluble brain protein kinase described by Miyamoto et al. (1). The microtubule preparation serves as an endogenous substrate for this protein kinase; both 6S and 30S tubulin are substrates for phosphorylation to the extent of about 0.10 ± 0.05 moles/mole.

INTRODUCTION

Purified microtubule protein (2) has been reported to have an associated protein kinase activity and to serve as a substrate itself for phosphorylation (3-8). The protein as isolated contains about 0.8 moles of serine phosphate per 110,000 daltons (9), and 2 moles of associated GTP (10). Despite numerous examples of cellular events which seem to involve both cyclic nucleotides and microtubules (e.g. 11), a physiological role for tubulin phosphorylation has not been established.

Recently (12-14), microtubules prepared from mammalian brain which can repolymerize in vitro have been described. In this study, a cAMP-stimulated protein kinase, distinct from tubulin, was found to be associated with the polymerization-competent preparation; it catalyzed a low level of phosphorylation in vitro of both tubulin 30S aggregates and of the tubulin 6S dimer. The possible significance of these findings to microtubule function is discussed.

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Abbreviations used: DEAE: diethylaminoethyl; cAMP: adenosine-3',5'-cyclic monophosphate; SDS: sodium dodecyl sulfate; ATP: adenosine 5'-triphosphate; TCA: trichloroacetic acid; EGTA: ethylene glycol-bis(β-aminoethylether)N,N'-tetraacetic acid; GTP: guanosine 5'-triphosphate; MES: 2(n-morpholino)ethane sulfonic acid.

Methods

Materials. Except where indicated, chemicals were obtained from Sigma.

Microtubule preparation. Fresh brains from male Wistar mice (>35g) were homogenized with 10 passes of a glass-teflon tube at 1000 rpm in one volume of assembly buffer (0.1M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.5) at 0°. Two cycles of polymerization-depolymerization were carried out according to ref. (14). Final pellets were allowed to depolymerize for 2-3 hr at 0° prior to further study.

Analytical methods. Discontinuous polyacylamide gel electrophoresis in tubes was run at pH 9.7 in an ethanolamine-triethanolamine buffer system (15). Gels were stained with Coomassie blue and scanned at 600 nm. Molecular weights were determined by comparison of mobilities with standard peptides: lysozyme, egg albumin, catalase, phospharylase a, and bovine serum albumin. Radioactive gels were sliced every 2 mm with a handmade device and dissolved in 0.2 ml of H_2O_2 for 4 hr at 60°. Radioactivity was measured in 5 ml of a solution of 2,5-diphenyloxazole (PPO), toluene, and Beckman solubilizer BBS-3.

Colchicine binding was measured by a DEAE-cellulose paper disc method (2).

Protein kinase activity was assayed (1) as TCA-precipitable ^{32}P obtained from [γ-^{32}P]ATP (5-11 Ci/mmole, New England Nuclear). Kinase containing samples of up to 0.175 ml were added to 0.025 ml of buffer containing 0.6 mg of calf thymus histone and 0.1 nmole of [γ-^{32}P]ATP with or without 1.0 nmole of cAMP. In experiments measuring endogenous substrate in column fractions, 0.085 ml of substrate fraction was added to 0.090 ml of kinase solution and 0.025 ml of the ATP solution. The reaction mixture was incubated for 5 min at 30° and then treated as described in ref. (1).

Protein was assayed (16) with the Folin reagent (Fisher) using crystalline bovine serum albumin as standard.

Results

Microtubules were purified from the crude supernatant fraction of mouse brain by two cycles of polymerization (12). The product obtained (yield: 1 mg/g wet tissue) bound 0.4 moles of [^3H] colchicine per 120,000 daltons with a K_D of 0.65 μM. Polyacrylamide gel electrophoresis in 0.1% SDS and 6M urea was performed on this preparation and a scan of Coomassie blue staining is shown in Fig. 1. The two major bands at 50,000 and 58,000 molecular weight are considered to be the two tubulin subunits (7, 13). The high molecular weight peptides (over 250,000 daltons), are reproducible; they might include dynein (17, 18).

When the microtubule preparation was incubated for 5 min at 30° with [γ-^{32}P]ATP, TCA-precipitable phosphate (0.2 $\pm$ 0.1 nmole/mg protein) was obtained. Addition of 5 μM cAMP increased phosphorylation several fold to 1.5 $\pm$ 0.5 nmole/mg protein (0.14 moles/120,000 daltons), which could not be increased by varying incubation conditions. The dotted trace in the SDS gel electrophorogram shown in Fig. 1 shows the ^{32}P-labeled peptides. Approximately

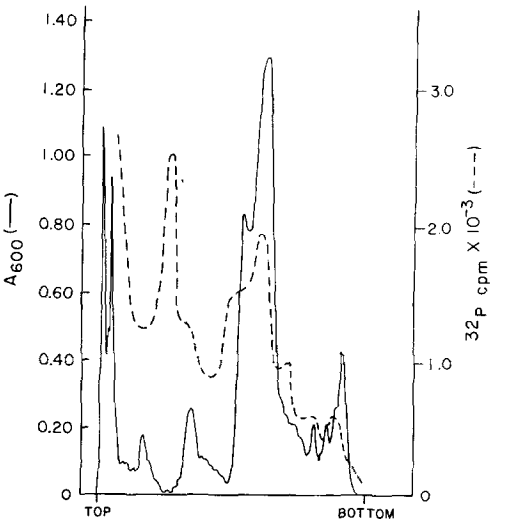


Figure 1. Polyacrylamide gel electrophoresis of polymerization-purified mouse brain microtubules. The sample was boiled for 2 min in ethanolamine-triethanolamine (15) pH 9.7, containing 1% SDS, 6M urea, and 10 mM β-mercaptoethanol and electrophoresed toward the anode in 7% gels containing 0.1% SDS and 6 M urea. Solid line: Coomassie blue stain A_{600} nm; dotted line: ^{32}P obtained as TCA-precipitable product of endogenous phosphorylation from [γ-^{32}P]ATP.

30% of the ^{32}P migrated with the two major protein bands (tubulin). High molecular weight proteins were also labeled.

The microtubule preparation was subjected to molecular sieve column chromatography on Sepharose 6B as shown in Fig. 2a (solid lines). The second peak of UV absorbance had an apparent size of about 120,000 daltons, while the first peak was apparently greater than 2 million. The third peak consisted mainly of guanine nucleotides (A_{260}/A_{280}, 1.45), present in the preparation but not in the column, but also contained trace amounts of some small proteins as seen in gel electrophoresis (not shown). Both peak I (0.04 moles) and peak II (0.1 mole/120,000 daltons) bound colchicine.

Gel electrophoresis of the pooled peaks I and II from Fig. 2a is shown in Fig. 3. The first peak resembles the total preparation (Fig. 1), with perhaps an enrichment in high-molecular weight peptides, and is primarily tubulin. The second peak of the column contains virtually pure tubulin, with the two subunits partially resolved.

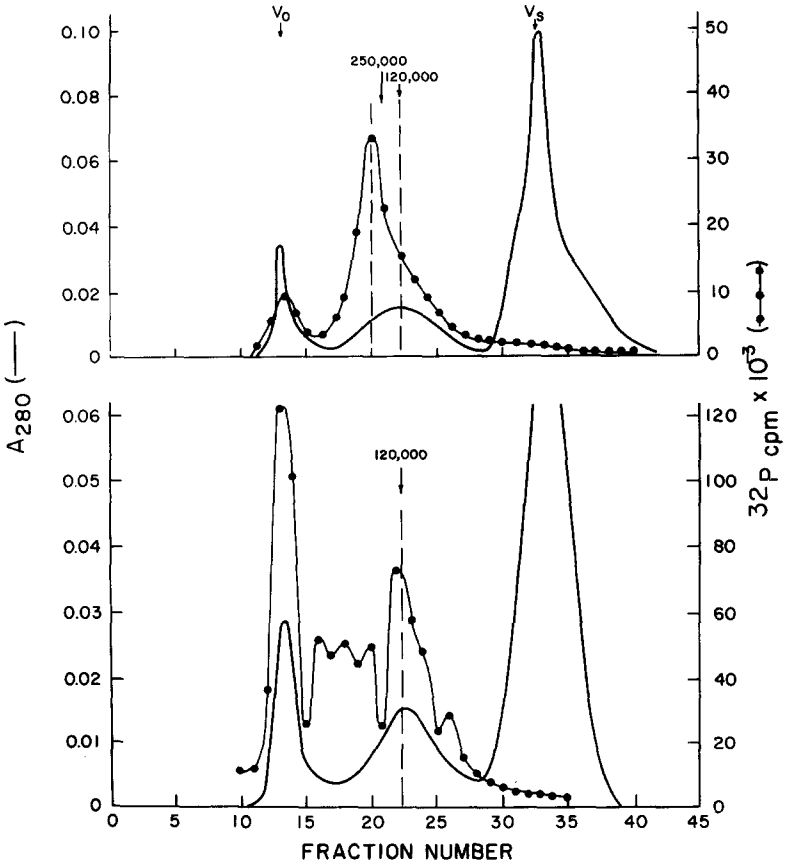
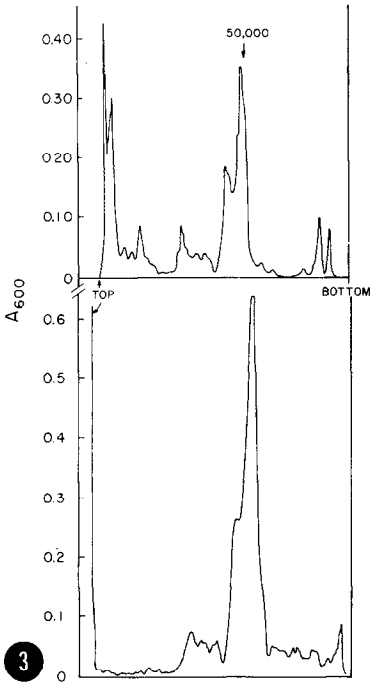


Figure 2. Sepharose 6B column chromatography of polymerization-purified mouse brain microtubules. Columns, 2.5 x 40 cm, were equilibrated with 50 mM Na-β-glycerophosphate, 0.6 mM EGTA, and 12 mM $Mg(OAc)_2$, pH 6.5 at 2°. The sample was 1.0 ml, 1.2 mg protein. The solid line in both A and B represents A_{280}. Vo and Vs represent the void volume and the salt volume. A_{280} material at Vs is mainly guanine nucleotides. The dotted line in part A represents endogenous protein kinase activity for exogenous substrate. In part B the dots represent endogenous substrate activity for protein kinase (tubes 17-19 in "A").

When aliquots of the Sepharose 6B fractions were assayed for kinase activity toward exogenous histone as substrate (Fig. 2a), some activity was found in the void volume, corresponding to the first UV-absorbing peak. A larger peak of activity was found eluting somewhat later, but ahead of the tubulin dimer peak. The kinase had an apparent molecular weight of 280,000 ± 30,000, based on the elution volume of proteins of standard molecular weight (kinase was slightly earlier to elute than catalase). It was definitely sep-



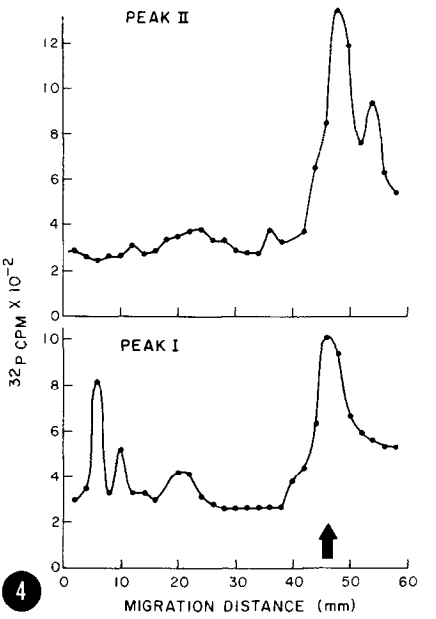


Figure 3. Polyacrylamide gel electrophoresis in SDS-urea of polymerization-purified mouse brain microtubules fractionated by gel filtration. Coomassie blue stain is determined as A_{600}. The upper trace represents the large molecular weight first peak (fractions 12-14) of Fig. 2; the lower trace represents the second UV-absorbing peak of about 120,000 molecular weight (tubes 20-25).

Figure 4. Polyacrylamide gel electrophoresis in SDS-urea of polymerization-purified mouse brain microtubules fractionated by gel filtration and then phosphorylated with [γ-^{32}P]ATP and protein kinase (tubes 17-19 of Fig. 2A.) The bottom trace represents the first peak of protein (pooled fractions 12-14 of Fig. 2B) and the upper trace represents the second peak (fractions 22-24 of Fig. 2B). Only ^{32}P radioactivity is shown, because protein quantities applied were too low for stain detection. The large arrow represents the position of Coomassie blue stain of a single major band observed in a parallel gel containing 50 µg of purified tubulin (fractions 22-23 of Fig. 2A).

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arated from microtubule protein and the tubulin dimer apparently does not possess protein kinase activity itself.

Using a separate but identical Sepharose 6B column, aliquots of each fraction were assayed for substrate capacity for the protein kinase obtained in the previous column (Fig. 2a fractions 17-19). Fig. 2b shows that TCA-precipitable ^{32}P was incorporated into fractions corresponding to the void volume UV absorbance peak and also into fractions peaking with the second UV

absorbance peak (6S tubulin). There is some substrate activity in column fractions between 6S and 30 tubulin; this phosphorylation is more significant on a weight basis than that of tubulin and indicates potentially interesting proteins in this preparation.

cAMP increased ^{32}P incorporation into all fractions which served as substrate. The amount of TCA-precipitable phosphate incorporated into both major peaks of substrate was approximately 0.1 mole of ^{32}P/120,000 daltons. Polyacrylamide gel electrophoresis in SDS-urea showed that substantial amounts of the ^{32}P migrated with tubulin in both cases, (Fig. 4). Other peptides are apparently phosphorylated as well. Tubulin is clearly a substrate for phosphorylation by cAMP-stimulated protein kinase.

Although the protein kinase represents a small amount of the protein in the polymerization-purified microtubule preparation, as shown by Fig. 2a, the proportion of kinase to tubulin is roughly constant through polymerization. When a 4X-polymerized preparation was chromatographed on the Sepharose 6B column, the protein kinase activity in the peak (fraction 20), showed the same proportion to the UV absorbance in the tubulin peak (fractions 18-26) as did the 2-cycle preparation: the 2-cycle preparation (Fig. 2) gave 2.2 pmoles/min/mg of protein kinase activity and 0.64 total A_{280} units while the 4-cycle material had 1.3 kinase units and 0.47 A_{280} units. The yield of kinase relative to tubulin was >80%.

The protein kinase from microtubule preparations consisting of fractions 17-20 from Fig. 2a, was stimulated about 7-fold by cyclic AMP, preferred histone to casein (10X) as substrate, required Mg^{2+}, and had an activity in the original preparation of 56 pmoles/min/mg with cAMP.

DISCUSSION

Purified microtubule protein has been reported to have autokinase activity which was stimulated by cAMP, as well as substrate capacity for exogenous protein kinase (3). The extent of tubulin phosphorylation was, however, only about 1% on a molar basis, and tubulin phosphorylation did not increase sig-

nificantly *in vivo* in cells stimulated to raise intracellular cAMP levels (4). Purified tubulin was reported (5) to have relatively high amounts of cAMP independent protein kinase activity towards exogenous substrate; this seemed to resemble a cAMP-regulated kinase which had lost its regulatory subunit. Incubation of tubulin with ATP and cAMP was reported to produce phosphorylated high molecular weight aggregates (19) which contained tubulin (7). Aggregation could have been due to phosphorylation or the conditions used (warming) (7). Since the protein was phosphorylated only to a low degree, probably on only one subunit, and not on any specific residue (7), it seemed that the *in vitro* phosphorylation of tubulin was not relevant to *in vivo* regulatory mechanisms. The protein kinase associated with tubulin through DEAE-Sephadex column purification was not stimulated by cAMP (7, but see 3). Isolated tubulin does contain 0.8 moles of one unique serine phosphate (8, 9). These results suggest no clear link between protein kinases, cAMP, and microtubule function.

We report here that polymerization-purified microtubule preparations also have an associated protein kinase, and that this activity is stimulated by cAMP. The cAMP-stimulated protein kinase is in constant proportion to tubulin through 2-4 polymerizations, suggesting a functional significance. A protein kinase was recently reported (20) for a polymerization preparation; those workers found that sedimentable microtubule polymers were not phosphorylated *in vitro* on the tubulin peptides. We find that the tubulin peptides are phosphorylated to the extent of about 0.1 moles by the endogenous protein kinase. However, several peptides in this preparation can be phosphorylated. The significance of tubulin phosphorylation *in vitro* to cellular events *in vivo* remains a question.

Microtubules are likely to be regulated with respect to polymerization competency, type of function, and cellular location; phosphorylation is one attractive possible mechanism for this regulation. It is still uncertain whether 30S differs from 6S tubulin (21) with respect to any chemical modifi-

cation, such as phosphorylation state. Studies seeking to analyze this question are in progress. The 30S and 6S tubulin appear to be equally competent substrates for protein phosphorylation *in vitro*.

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