ENDOGENOUS PHOSPHORYLATION AND DEPHOSPHORYLATION OF MICROTUBULE-ASSOCIATED PROTEINS ISOLATED FROM BOVINE ANTERIOR PITUITARY

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1. Introduction

Previous reports [1,2] have shown that tubulin, prepared by ion exchange chromatography from mammalian brain [3], can be phosphorylated by a closely associated cyclic AMP-dependent protein kinase. Phosphorylation of mammalian brain tubulin has also been shown to occur in slices [4] and in vivo preparations [5]. However, the amount of phosphate incorporated into tubulin suggested that only a small proportion of the tubulin molecules were phosphorylated. Recently a cyclic AMP-dependent protein kinase has been found in microtubule proteins isolated from mammalian brain [6] by an in vitro assembly–disassembly procedure [7] but in these preparations the phosphate was predominantly incorporated into microtubule associated proteins of higher molecular weight than tubulin.

In this report we describe the preparation of microtubule proteins from a hormone secreting tissue by an in vitro assembly method [7]. This preparation shows rapid, cyclic AMP-dependent, incorporation of [^{32}P] phosphate from γ-[^{32}P] ATP into several components, two of which comigrate with minor proteins of mol. wts 7×10^4 and 28×10^4 on SDS gel electrophoresis. In contrast to the preparations from mammalian brain, however, the preparation also contains an active phosphatase which rapidly dephosphorylates the endogenous substrates and is also active on exogenous phosphorylase *a*. The occurrence of both a protein kinase and a protein phosphatase in pituitary microtubule preparations would be consistent with regulation of microtubule function in this tissue by a phosphorylation–dephosphorylation cycle.

2. Materials and methods

2.1. *Materials*

Chemicals were obtained from either BDH (Chemicals) Ltd., Poole, Dorset, U. K. or Boehringer Corp. (London). Ltd. Bell Lane, Lewes, U. K. Carrier free [^{32}P] orthophosphate was purchased from the Radiochemical Centre, Amersham, Bucks, U. K. Butyl-PBD [5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa-3, 4-diazole] was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, U. K.

2.2. *Preparation of γ-[^{32}P] ATP*

γ-[^{32}P] ATP was prepared by a modification of the method of Glynn and Chappell [8], to an initial specific radioactivity of 790 μCi/μmol. The ATP was stored at −14°C in 0.8 M Tris–Cl pH 7.6. The specific radioactivity of the ATP was measured using a luciferase/charcoal method [9].

2.3. *Preparation of microtubular proteins*

Microtubular proteins were isolated essentially by the method of Shelanski et al. [7]. Bovine anterior pituitary glands were obtained within 5 min of the animal's death, minced with scissors, and homogenised, using a Polytron, in an equal volume of ice-cold 20 mM sodium phosphate pH 6.8 containing 2 mM $MgCl_2$, 2 mM EGTA and 1 mM GTP. The homogenate was centrifuged at 120 000 g_{av} for 75 min at 4°C. The supernatant, which contained most of the tubulin [10], was added to an equal vol. of 8 M glycerol containing the same buffer and incubated at 37°C for 30 min to allow polymerisation of microtubule proteins. The microtubules were sedimented at 100 000 g_{av} for 60 min at 37°C. The pellet was gently washed

with warm 50 mM Tris–Cl pH 7.6, then resuspended using a teflon pestle in the same buffer. The microtubules were depolymerised on ice for 20 min; any insoluble material remaining after this time was removed by centrifugation at 15 000 g_{av} for 2 min on an Eppendorf 3200. The preparation could be further purified by repetition of the assembly–disassembly cycle.

2.4. *Phosphorylation of endogenous substrates*

Tubes were prepared containing about 25 μg of microtubular protein in 100 mM Tris–Cl pH 7.6 containing 100 μM $MgCl_2$, and, where appropriate, cyclic AMP (10 μM) and/or sodium fluoride (50 mM). γ-[^{32}P]-ATP was added, to a final concentration of 100 μM in a final vol. of 50 μl, to start the reaction. Tubes were incubated at 37°C for the times given; then, to measure bound phosphate, duplicate aliquots (15 μl) were removed and layered onto rectangles (2 × 1 cm) of Whatmann 3MM paper, which were immediately immersed in ice-cold 10% (w/v) TCA.

To determine the amount of liberated [^{32}P] phosphate, Norit A charcoal (5 mg) in 5% v/v perchloric acid containing 1 M sodium phosphate was added to the remaining incubation mixture, to adsorb any γ-[^{32}P] ATP. After sedimentation of the charcoal an aliquot of the supernatant was assayed for radioactivity in the presence of 5 ml scintillator [600 ml toluene, 400 ml methoxyethanol containing 80 g napthalene and 6 g butyl-PBD).

The paper rectangles were washed for twenty minutes in two changes each of ice-cold 10% w/v TCA and then ice-cold 5% w/v TCA, followed by a final wash in cold acetone. The papers were shaken dry and assayed for radioactivity in the presence of 5 ml butyl-PBD scintillator.

2.5. *SDS gel electrophoresis*

SDS gel electrophoresis was performed essentially as described by Weber and Osborn [11]. Samples were were incubated at 95°C for 15 min in the presence of 10 mM sodium phosphate pH 7.0 containing 2% w/v SDS, 5% v/v β-mercaptoethanol, 10% sucrose and 0.02% bromophenol blue. Samples were then layered onto 10 cm gels (4.5% w/v acrylamide, 0.12% w/v methylene bis-acrylamide) and run at 6–8 mA per tube. The gels were strained with Coomassie brilliant blue and scanned using a Unicam SP500 with a Gilford scanning attachment.

To measure bound radioactivity in the gels, they were sliced into 2 mm sections, these were dissolved in 100 volume hydrogen peroxide (300 μl) at 95°C for 2 hr and the excess hydrogen peroxide evaporated off. The residue was dissolved in 100 μl of 0.1 M HCl and assayed for radioactivity in the presence of 5 ml butyl-PBD scintillator.

Mol. wt determinations were made by comparing the mobility of the unknown with standard samples; myosin, phosphorylase, pyruvate kinase, brain tubulin and lactate dehydrogenase.

3. Results

In the presence of γ-[^{32}P] ATP, microtubule proteins (0.5 mg/ml) rapidly incorporated [^{32}P] phosphate into TCA insoluble substrates (fig.1a). Cyclic AMP (10 μM) approximately doubled both the rate of phosphate incorporation into the TCA precipitated protein and the final amount of phosphate incorporated. In the experiments shown in fig.1, the phosphate incorporated reached a maximum at 4 min and remained constant up to 12 min. In other experiments using microtubule proteins at concentrations greater than 1 mg/ml (data not shown), phosphate incorporation was maximal within 1–2 min and decreased rapidly thereafter, suggesting that in addition to a kinase, the preparation contains a protein phosphatase and can rapidly hydrolyse the ATP present. To demonstrate protein phosphatase activity, microtubule proteins (0.5 mg/ml) were phosphorylated for 4 min, and the ATP then removed by addition of sufficient hexokinase and glucose to convert the ATP to glucose 6-P and ADP within 5 sec. On addition of hexokinase, the radioactivity in the TCA precipitate decreased by more than 50% within 30 sec. (fig.1a, dotted line). To demonstrate ATPase activity, radioactive ATP was adsorbed from an aliquot of the reaction mixture by addition of charcoal and the supernatant radioactivity, which represents free [^{32}P] phosphate, determined in the experiments shown in fig.1a. Approximately 50% of the initial [^{32}P] ATP was hydrolysed to [^{32}P] phosphate within 4 min and cyclic AMP did not alter the rate of hydrolysis (fig.2a).

The amount of phosphate present in the TCA

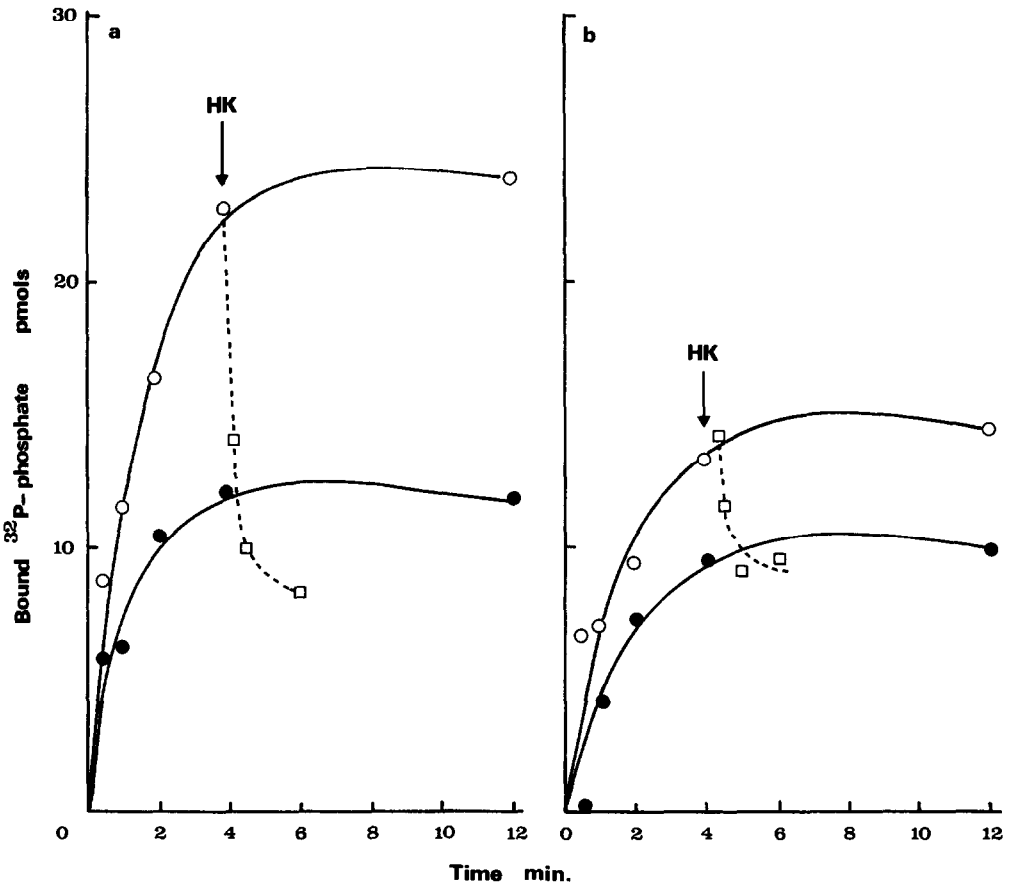

Fig.1,a,b. Time course of phosphorylation of endogenous microtubule substrates. Microtubule proteins (25 μg) were prepared and incubated for the times shown as described in the Methods section. [^{32}P] phosphate incorporation was determined by the filter paper method described. In one experiment hexokinase (3 μg) and glucose (13 μg) were added at 4 min and the TCA insoluble [^{32}P] phosphate determined at various times after the addition. (a) (•) Control. (○) Cyclic AMP (10 μM). (■) After addition of hexokinase and glucose to the cyclic AMP incubation. (b) as in fig.1a but in the presence of 50 mM sodium fluoride. Each point is the mean of duplicate determinations on two microtubule preparations.

precipitate represented only a small proportion of the total phosphate liberated from ATP, but the observed ATPase activity could result from the rapid turnover of this protein-phosphate catalysed by a protein kinase and a protein phosphatase. Fluoride is known to inhibit several phosphatases, and at 50 mM it partially inhibited dephosphorylation of phosphorylase *a* by the phosphatase present in the microtubule preparation fig.3. As expected if the ATPase activity involved this phosphatase, fluoride also partially inhibited the liberation of [^{32}P] phosphate from ATP by the microtubule preparation (fig.2b). If this were the only action of fluoride in the system, an increased incorporation of phosphate into protein would be expected. However, fluoride decreased the rate of phosphate incorporation and the final concentration of phosphate incorporated into TCA-insoluble material (fig.1b). The inhibition was most marked in the presence of cyclic AMP. Endogenous phosphatase activity was not completely inhibited by fluoride; addition of hexokinase and glucose at 4 min resulted in a small decrease in protein phosphate concentration (fig.1b dotted line).

Samples of the reaction mixture were quenched, by addition of TCA, and were prepared for gel electrophoresis. The staining pattern revealed a major band

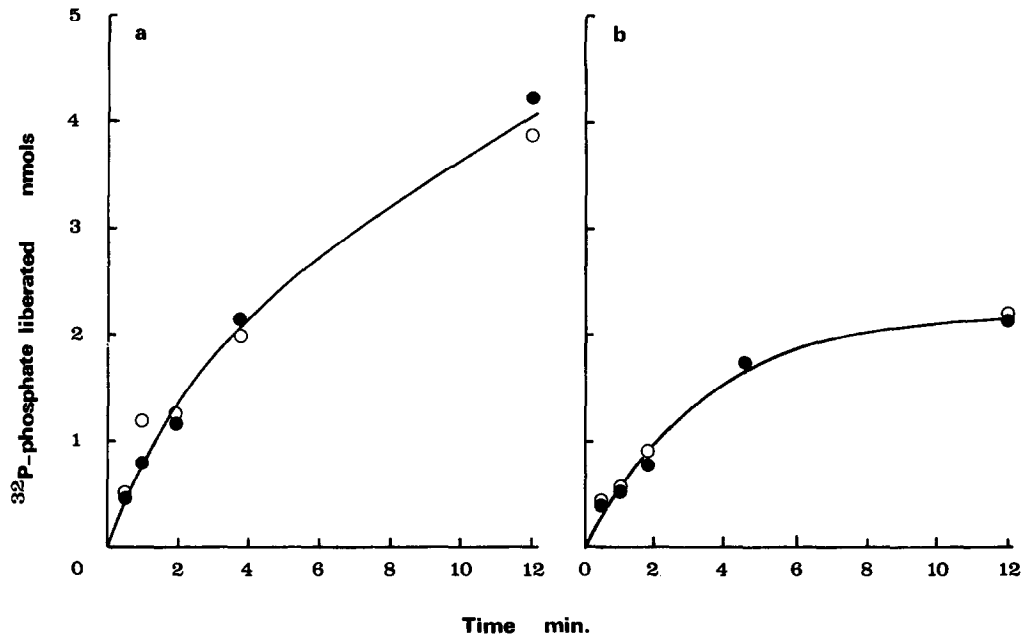

Fig.2,a,b. Time course of microtubule ATPase activity. Microtubule proteins were incubated as described in the legend of fig.1. Free [^{32}P] phosphate was determined after adsorption of any remaining γ-[^{32}P] ATP onto charcoal. (a) (•) Control. (○) Cyclic AMP (10 μM). (b) As in fig.2a but in the presence of 50 mM sodium fluoride. Points are the mean of values obtained from two different experiments.

which comigrated with brain tubulin [3] and several associated bands notably of mol. wts 280 000, 70 000, 64 000 and 45 000 which constitute less than 10% of the total protein (fig.4a,b). In the absence of cyclic AMP, [^{32}P] phosphate was incorporated largely into the 70 000 mol. wt component (fig.4c). Cyclic AMP stimulated incorporation of [^{32}P] phosphate into several components of the microtubule preparation, including the 70 000 and 280 000 mol. wt components and tubulin itself. On a molar basis tubulin only incorporated about 10% of the phosphate bound to the higher molecular weight bands, in the presence of cyclic AMP.

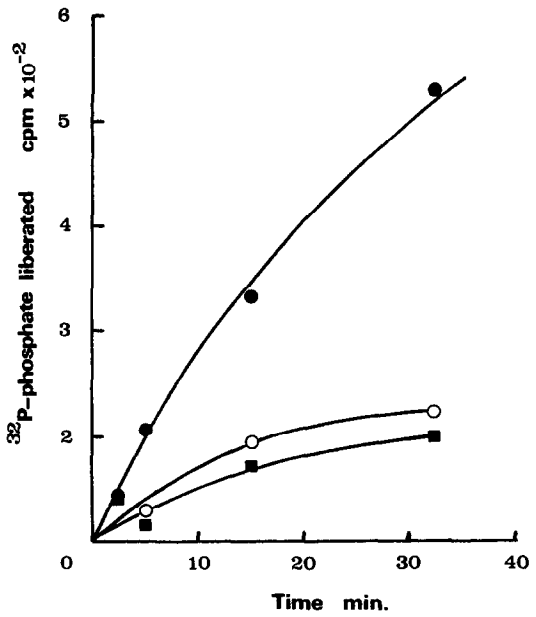

Fig.3. Dephosphorylation of phosphorylase *a* by microtubule phosphatase. [^{32}P] phosphorylase *a* (8 μg) was incubated with samples of microtubule proteins, prepared as described in the Methods section, in 50 mM Tris–Cl pH 7.6 containing 2 mM $MgCl_2$ (100 μl) at 37°C. The reaction was quenched by the simultaneous addition of 100 μl bovine serum albumin (10 mg/ml) and 200 μl 10% w/v TCA. The precipitated proteins were sedimented for 2 min at 15 000 g_{av} on an Eppendorf 3200. An aliquot of the supernatant was assayed for radioactivity. (•) Control. (○) 50 mM Sodium fluoride. (■) 100 mM Sodium fluoride.

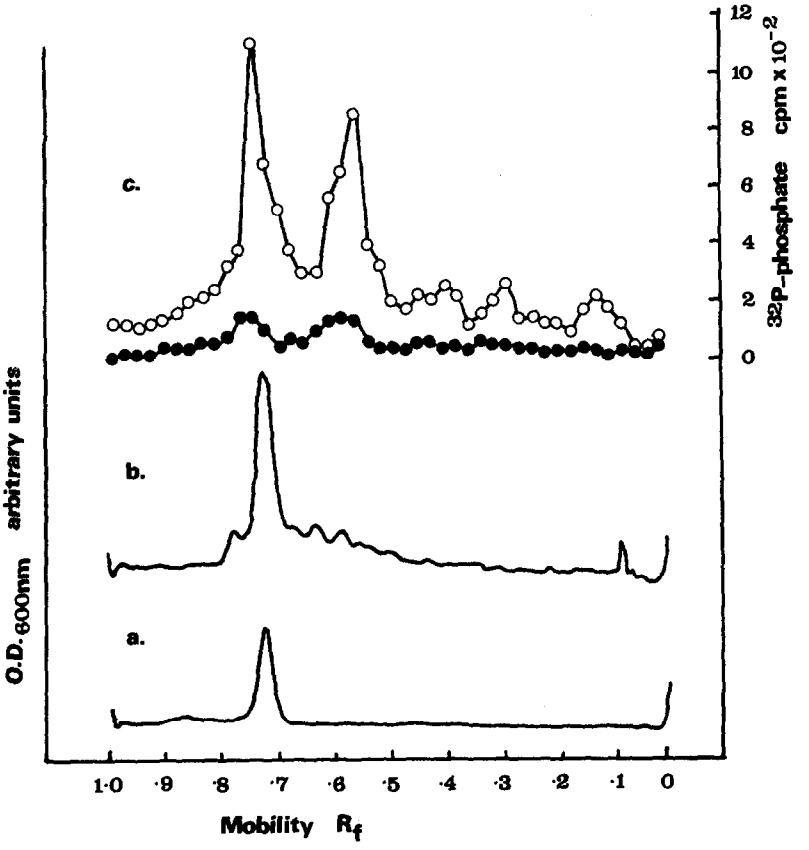

Fig.4. Distribution of [^{32}P]phosphate and proteins on SDS gel electrophoresis. Microtubule proteins were prepared and incubated for 4 min as described in the methods section. Samples were then quenched by the addition of 100% TCA to a final concentration of 10% w/v. The precipitate was washed in 10% TCA, then dissolved in SDS, run and stained as described in Materials and methods. (a) Brain tubulin (20 μg) prepared by ion-exchange chromatography [3]. (b) Once polymerised pituitary microtubule preparation. (c) [^{32}P]phosphate in 2 mm sections of gel, at the same concentration as in 4b, in the absence (•) and presence (○) of 10 μM cyclic AMP. (ATP specific radioactivity 145 μCi/μmol).

4. Discussion

High mol. wt proteins associated with brain microtubules assembled in vitro have been shown to contain ATPase activity [13–15]. The microtubule proteins isolated from bovine anterior pituitary also exhibit rapid ATPase activity, which could be catalysed by an intrinsic cyclic AMP dependent protein kinase and a protein phosphatase acting through an endogenous substrate. The protein phosphatase present in this preparation is also active on an exogenous substrate phosphorylase *a*, and, like other phosphatases, can be inhibited by fluoride. The inhibitory effect of fluoride on the incorporation of phosphate into the endogenous substrates suggests that fluoride also inhibits the kinase. This overall reduction in the rate of the kinase/phosphatase cycle by fluoride is paralleled by a decreased rate of ATP hydrolysis, which is consistent with this hypothesis. The independence of the overall ATPase on cyclic AMP presumably means that the phosphatase is rate limiting.

The evidence does not however preclude the possibility that some of the phosphate incorporated into TCA-incoluble material is present as a stable phosphorylated intermediate of an ATPase, similar to the calcium regulated ATPase from sarcoplasmic reticulum [16] or the Na^+/K^+ ATPase [17]. However, the microtubule ATPase activity is unaffected by

incubation with 0.5 mM EGTA, 1 mM calcium, 4 mM sodium azide, and 1 μM ouabain (data not shown).

The high mol. wt proteins, which are also present in brain microtubule preparations, appear to constitute the filamentous projections seen on in vitro polymerised microtubules [15]. These proteins are also similar to the high mol. wt proteins, the dyneins, found associated with flagellar microtubules, and which are thought to mediate the motile force. There is evidence to suggest that microtubules are involved in the release of growth hormone from the anterior pituitary [10]. It is interesting to speculate whether the dependence of the release process on ATP and its sensitivity to changes in cyclic AMP content [18], derive from the requirements of this system.

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