Presence of two distinct adenosine triphosphatase activities in bovine brain microtubules

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1. INTRODUCTION

Axonal transport in neuronal tissues is an energy-dependent process requiring the hydrolysis of ATP [1–3], and it has been assumed that a dynein-like ATPase may be associated structurally with brain microtubules. Although there have been several reports which point to the presence of an ATPase activity in preparations of brain microtubules [4–9], it was not certain whether these ATPases were specifically associated with any of the components of microtubules.

An ATPase activity is present in the fraction of microtubule-associated proteins (MAPs) from rat brain and is stimulated by the addition of purified 6 S tubulin in the presence of Ca^{2+} [10]. The dependency on tubulin of the ATPase activity suggested its structural and functional association with microtubules.

Here on further purification of the ATPase activity from the MAPs fraction of bovine brain microtubules, 2 distinct ATPases are separated, designated ATPase 'I' and 'II'. ATPase I is of relatively low M_r ($\sim$33 000) and dependent on added tubulin in the presence of Ca^{2+}. On the other hand, ATPase II has a larger size and appears to be associated with membrane vesicles. It is observed under electron microscopy as membrane vesicles containing knob-like structures reminiscent of H^+-pump ATPases. A preliminary account of this paper appeared in [11].

Abbreviations: MES, 2-(*N*-morpholino) ethane sulfonic acid; Tris, tris (hydroxymethyl) aminomethane; EGTA, ethyleneglycol-bis(2-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid

2. MATERIALS AND METHODS

Phosphocellulose (P 11) and DEAE-cellulose (DE 52) were obtained from Whatman. Pepstatin was a kind gift of Dr Matsushima. ^{32}p-Labeled orthophosphate was purchased from New England Nuclear, and [γ-^{32}P]ATP was prepared as in [12], and purified on a Dowex 1 ($\times$2) column [13].

Twice-cycled microtubules were prepared from bovine brain by successive cycles of temperature-dependent assembly-disassembly according to [14], with some modifications. Tubulin was prepared from twice-cycled microtubules by phosphocellulose column chromatography [10]. Tubulin was measured either by [^{3}H]GTP binding assay as in [15], or by [^{3}H]colchicine binding assay [16] using a modification of the rapid gel filtration method in [17].

The standard assay for ATPase activity contained in 50 μl; 20 mM MES-K (pH 6.5 at 20°C), 5 mM 2-mercaptoethanol, 0.4 mM magnesium acetate, 5% (v/v) glycerol, 0.3 mM [γ-^{32}P]ATP and 1.1 μM tubulin. Further additions were 10 mM $CaCl_2$ for ATPase I and 20 mM KCl for ATPase II, respectively. After incubation for 20 min at 37°C, liberated P_i was determined [13]. Protein concentrations were determined by the colorimetric method [18], using bovine serum albumin as a standard.

Samples of ATPase II were applied onto Formvar membrane-coated copper grids (300 mesh). Staining was accomplished by the sequential addition of a drop of the sample solution and 1.5% aqueous solution of uranyl acetate. The grids were examined with an H-500 electron microscope (Hitachi Co., Tokyo) at $\times$50 000.

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Table 1

Association of ATPase activity with rat brain microtubules

Fraction[a]	Total protein (mg)	Total activity (units)[b]	Specific activity (units/mg)
S-100	334	636	1.9
Sup-1	245	233	1.1
MTS-1	17	144	8.5
Sup-2	5.2	21	4.0
MTS-2	7.0	79	11.3
Sup-3	0.7	4.1	5.9
MTS-3	4.8	63	13.1

[a] S-100 is a 100 000 × *g* supernatant fraction of rat brain homogenate. Sup-1 and MTS-1, Sup-2 and MTS-2, and Sup-3 and MTS-3 are the supernatant and the precipitate fractions, respectively, of cycles 1,2 and 3 of assembly

[b] One unit is defined as 1 nmol ATP hydrolyzed/min at 37°C

Microtubules were purified by temperature-dependent cycles of assembly–disassembly [14] and the distribution of ATPase activity and protein in the supernatant and the precipitate were measured at each cycle

3. RESULTS

Table 1 shows that the ATPase activity was found to copurify with microtubules prepared from rat brain through multiple cycles of temperature-dependent assembly–disassembly. The non-specific ATPase activity present in the crude extracts was eliminated mostly in the first supernatant fraction (Sup-1), and the specific activity of the ATPase in microtubule fraction approached to a constant level after 2 (MTS-2) or 3 (MTS-3) cycles of assembly. These results indicate the intimate association of the ATPase activity with brain microtubules.

To purify the ATPase activity further, the MAPs fraction was prepared from twice-cycled bovine brain microtubules by chromatography on a phosphocellulose column. The fractions eluted with 0.6 M KCl from the phosphocellulose column were combined, dialyzed, and applied to a DEAE-cellulose column. Two distinct ATPases were separated by this column chromatography (fig.1). ATPase I, which was eluted with 0.2 M KCl, was almost com-

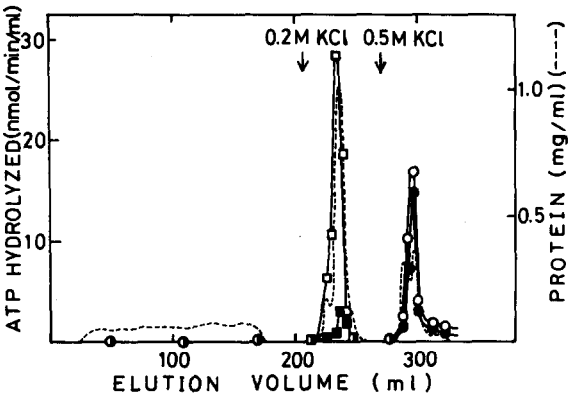

Fig.1. DEAE-cellulose column chromatography of microtubule-associated ATPases. The MAPs fraction (~34 mg protein) eluted with 0.6 M KCl from a phosphocellulose column was dialyzed against 20 mM Tris-HCl (pH 7.5 at 10°C), 5 mM 2-mercaptoethanol, 0.5 mM magnesium acetate, 0.1 mM EGTA, 10% (v/v) glycerol, 1 μg pepstatin/ml, 10 μM ADP, and was applied on a column (1.5 × 20 cm) of DEAE-cellulose. The column was washed with the above buffer and eluted with 0.2 M KCl and succeedingly 0.5 M KCl. ATPase I activity was measured with (□) or without (■) 1.1 μM tubulin in the presence of 10 mM $CaCl_2$. ATPase II activity was measured with (○) or without (●) tubulin in the absence of Ca^{2+}; (---), [protein].

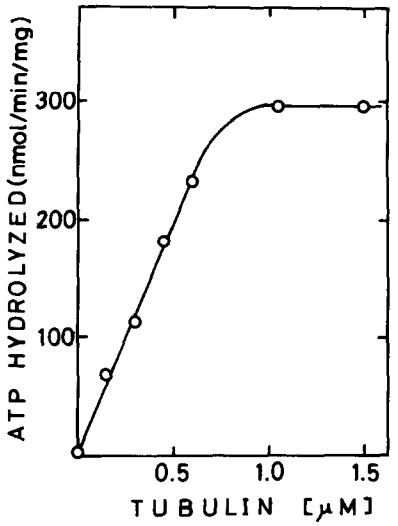

Fig.2. Dependency of the ATPase I activity on tubulin. ATPase activity was assayed in the presence of various [tubulin] and 10 mM Ca^{2+} as in section 2.

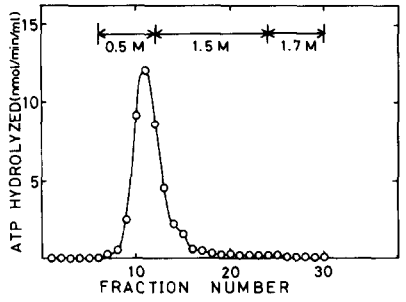

Fig.3. Sedimentation profile of ATPase II on a discontinuous sucrose density gradient. ATPase II in 20 mM Tris–HCl (pH 7.5 at 10°C), 5 mM 2-mercaptoethanol, 0.5 mM magnesium acetate, 0.1 mM EGTA, 10 μM ADP, 1 μg pepstatin/ml and 10% (v/v) glycerol was applied on a discontinuous sucrose density gradient of 0.5 M, 1.5 M and 1.7 M sucrose dissolved in the above buffer. After centrifugation in a Spinco SW 50.1 rotor for 19 h at 50 000 rev./min and 2°C, the fractionated samples were assayed for ATPase II activity.

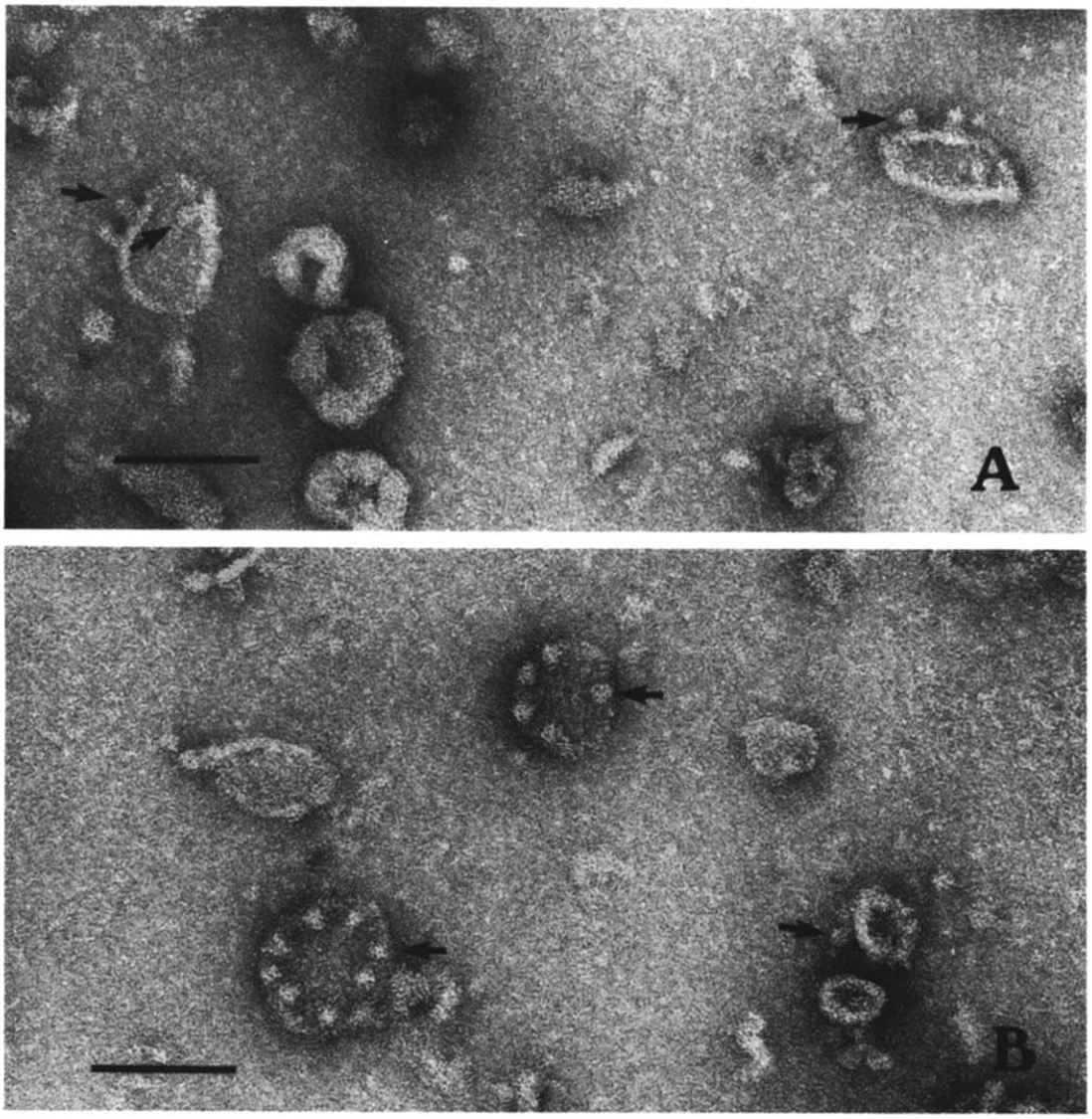

Fig.4. Electron micrographs of the purified preparation of ATPase II. The preparation of ATPase II purified through the phenyl–Sepharose column chromatography was negatively stained with 1.5% uranyl acetate. Knob-like protrusions on the vesicles are shown in the side-view (A) and the surface-view (B). The bars correspond to 100 nm.

pletely dependent on added tubulin in the presence of Ca^{2+}. However ATPase II, which was eluted with 0.5 M KCl, was not stimulated by added tubulin and rather inhibited by Ca^{2+} (not shown).

ATPase I was further purified by successive column chromatographies on CM-cellulose and Sephacryl S-300. The purified ATPase I was ~33 000 M_r as determined by gel filtration procedure. ATPase I was very unstable but could be stabilized by the addition of 0.1 mM ADP, 0.3 mg tubulin/ml or 50% (v/v) glycerol. ATPase II was purified by hydrophobic chromatography on phenyl–Sepharose. It had a larger size and sedimented in the glycerol density gradient centrifugation with a sedimentation coefficient of 27 S. ATPase II was found to be associated with membrane vesicles (see below). The details of the purification and properties of ATPase I and ATPase II will be described elsewhere.

Fig.2 shows that purified ATPase I was completely dependent on tubulin in the presence of Ca^{2+}. The stimulation of the ATPase I activity by tubulin was not due to the result of its stabilization by tubulin [10]. The stimulation was specific to the native form of tubulin since preparations of tubulin denatured either by heating for 1 min at 90°C, or more mildly by dialysis in the absence of glycerol were completely ineffective. Divalent cations other than Ca^{2+} could not substitute Ca^{2+} for the stimulation of the ATPase I activity.

Since ATPase II was hydrophobic, and had a rather high *s*-value (27 S), we studied its sedimentation behavior on the discontinuous sucrose density gradient. ATPase II activity was recovered in the interface of 0.5 M and 1.5 M sucrose (fig.3). The electron microscopic examination of the preparation of ATPase II revealed that it consisted of membrane vesicles ranging from 70–100 nm diam. (fig.4). Knob-like protrusions of 16–20 nm diam., similar to those of H^+-pump ATPases in appearance, were observed on the outer surface of these vesicles. Oligomycin at 0.1 mM and ouabain at 0.1 mM did not inhibit the activity of ATPase II.

4. DISCUSSION

The above results indicate that two distinct ATPase activities are associated with brain microtubules. Both ATPase I and ATPase II copurified with microtubules through cycles of assembly and disassembly (table 1) suggesting their structural and functional association with brain microtubules. However, contrary to our expectation, none of them corresponded to any major protein components of the MAPs fraction.

The activity of ATPase I is nearly completely dependent on added tubulin in the presence of Ca^{2+}. Because of its high degree of instability, ATPase I constituted only a minor fraction of ATPase activity in the MAPs fraction (e.g., fig.2 in [11]). We have stabilized ATPase I activity (not shown) which then became comparable to that of ATPase II (fig.1). This may be why ATPase I had been missed by other workers.

A puzzling property of ATPase I is the requirement of an unphysiologically high $[Ca^{2+}]$ (~2 mM) for its maximal activation by tubulin. The addition of calmodulin from porcine brain did not lower the optimal $[Ca^{2+}]$, but inhibited ATPase I activity almost completely (not shown). This might be due to the interaction of tubulin with a Ca^{2+}–calmodulin complex [19,20]. We have no suitable explanation for the requirement of unphysiologically high $[Ca^{2+}]$ for ATPase I activity.

It is of interest that ATPase II, which is associated with membrane vesicles, copurifies with brain microtubules. Although the identity of the vesicles is not clarified yet, they were observed under electron microscope as vesicles of 70–100 nm diam, with knob-like structures protruding outside of the membrane vesicles. The contamination of mitochondrial H^+-pump ATPase was excluded because ATPase II activity was insensitive to 0.1 mM oligomycin. It has been reported that chromaffin granules and synaptic vesicles may possess structures similar to H^+-pump ATPase [21]. These properties of ATPase II are very similar to those of the ATPase isolated from brain microtubules in [22]. In [22] no description of the distinct ATPases was made. Probably, only the major species of ATPase was purified [22], and ATPase I lost because of its extreme instability. The identification of the vesicles and the significance of their association with brain microtubules must await further investigation.

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