A NEW ATPase IN CYTOPLASMIC MICROTUBULE PREPARATIONS

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1. Introduction

Microtubules, cylindrical structures of about 250 Å in diameter, are present in the cytoplasm of most eukaryotic cells. Although their functions are still obscure, there are grounds for believing that they play a significant role in intracellular motion. This follows, in particular, from the fact that the breakdown of microtubules (e.g., by colchicine) results in cessation of divergence of chromosomes in mitosis [1], of axoplasmic transport [2], of saltatory movement of intracellular granules [3] and of some other types of motion.

If microtubules directly participate in cytoplasmic transport, it is natural to assume that they contain ATPase whose role is mechanochemical coupling. Such ATPase is well known in the case of cilia and flagella: it is dynein, a protein with a mol. wt > 300 000, inherent in microtubules of axoneme outer doublets [4].

The present work demonstrates that microtubule preparation isolated from bovine brain by polymerization–depolymerization procedure contains ATPase. This ATPase is capable of cosedimentation with microtubules. Its activity is not suppressed by membrane ATPase inhibitors, neither is it due to the presence in the preparation of actin or myosin impurities. Possibly, the ATPase revealed is inherent to microtubules in vivo and concerned with mechanochemical coupling.

2. Methods

2.1. *Preparation of tubulin*

Tubulin was obtained from bovine brain by polymerization–depolymerization procedure [5] modified as in [6]. Instead of phosphate buffer as in [6], we used 10 mM imidazole–HCl buffer, pH 6.7 with 50 mM KCl and 0.5 mM $MgCl_2$ for homogenization of the tissue. For polymerization, the buffer was supplemented with glycerol to 4 M and ethyleneglycol-bis-(2-aminoethyl ester)-*N,N'*-tetra-acetate (EGTA) to 1 mM.

2.2. *Protein assay*

Protein was assayed spectrophotometrically assuming that $E^{1\%}_{275}$ for tubulin is 12 [7].

2.3. *Gel filtration*

Gel filtration of the preparation was done at 4°C on a 1.6 × 65 cm column with Sepharose 4B. The applied sample was vol. 2 ml, elution was at a rate of 0.2 ml/min and the fraction volume 2 ml.

2.4. *Determination of ATPase activity*

The mixture used for determination of ATPase activity contained in total vol. 0.5 ml (unless otherwise indicated) 0.4–0.5 mg protein and the following buffer components: 10 mM imidazole–HCl pH 6.7, 0.5 mM $MgCl_2$, 50 mM KCl, 1 mM ATP (standard buffer). Incubation was for 60 min at 37°C and the reaction was stopped by adding 0.5 ml $HClO_4$. The mixture was centrifuged and the inorganic phosphate concentration [P_i] in the supernatant determined calorimetrically [8]. Under the above conditions the dependence between the time of incubation and the amount of split ATP remained linear during 60 min.

3. Results

The depolymerized microtubule preparation isolated from bovine brain is characterized under standard conditions by ATPase activity of about 10 nmol·min^{-1}·mg^{-1}. The activity of the preparation significantly depends on the ionic composition of the incubation medium (table 1). For maintaining the enzyme activity, the presence of divalent cations is required: their removal with EDTA results in loss of activity. In the absence of Ca^{2+} ions, optimal conc. Mg^{2+} is 2–4 mM. The activity of the enzyme in the presence of 0.5 mM Mg^{2+} can be increased by 75% by addition of $CaCl_2$ to conc. 15 mM. High ionic strength inhibits the ATPase activity of the preparation. Table 1 shows that 0.2 M KCl decreased the activity by 78%, and 0.5 M KCl by 98%. The effect of high ionic strength on the ATPase activity is completely reversible.

The presence of ATPase activity in a tubulin preparation obtained by polymerization–depolymerization procedure indicates that the enzyme is capable of being polymerized and depolymerized under the same conditions as tubulin. However, this is hardly a direct proof of ATPase being inherent in microtubules. Therefore we carried out some more experiments to test the suggestion. In particular, we incubated a standard tubulin preparation in the presence of 4 M glycerol, 1 mM GTP and 1 mM EGTA, applied the assembled microtubules obtained onto 5 M glycerol and sedimented them by centrifugation.

Table 1
Effect of ionic strength on the tubulin preparation ATPase activity

KCl (mM)	ATPase act. (%)
50	100
150	64
200	36
250	22
500	2

The ATPase activity was determined in the medium with different KCl concentrations. The concentrations of the rest of the components were the same as in the standard buffer. The ATPase activity of the preparation in the standard buffer (50 mM KCl) was assumed to be 100% (this activity was 6.0–8.6 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$

The pellet obtained was suspended in a buffer, depolymerized and its activity was determined. It was found that sedimentation through 5 M glycerol failed to cause a decrease in the specific activity of the preparation.

Gel filtration of the tubulin preparation obtained by polymerization–depolymerization through a Sepharose 4B column is known to reveal two fractions [9]. The heavy fraction eluting in the void volume of the column contains primarily tubulin oligomers and minor components of the preparation. The light fraction contains highly purified 6 S tubulin. Analysis of the ATPase activity of the fractions after gel filtration (fig.1) demonstrates that all activity is eluted in the void volume of the column.

Thus the ATPase studied cosediments with microtubules during centrifugation through a layer of 5 M glycerol, and in a depolymerized preparation it is eluted together with tubulin oligomer in gel filtration. This may indicate that it is inherent to tubulin.

It is not excluded, however, that this association is not specific and that the ATPase activity of the preparation is due to non-specific adsorption of one of the known ATPases on microtubules. We tried to verify this suggestion using known membrane ATPase inhibitors. Table 2 illustrates the results of experiments on the effect of oligomycin and ouabain on the ATPase activity of the preparation. It can be seen that these inhibitors, in concentrations sufficient for

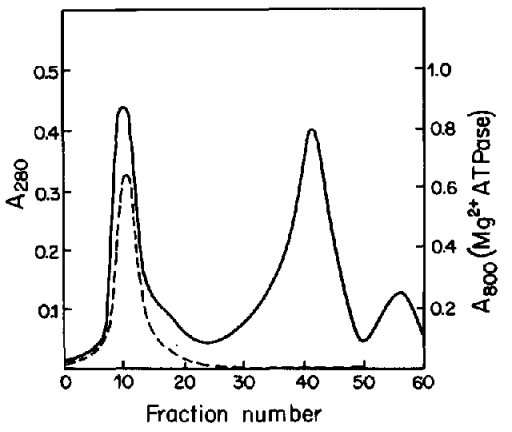

Fig.1. Gel filtration of the tubulin preparation on Sepharose 4B. Column vol., 130 ml; fraction vol., 2 ml; elution rate, 12 ml/h. (– – –) ATPase activity; (——) A_{280}.

Table 2
Effect of inhibitors on tubulin ATPase activity[a]

Inhibitor	ATPase act. (%)
None	100
Oligomycin (1 μg/ml)	97
Oligomycin (4 μg/ml)	92
Oligomycin (10 μg/ml)	93
Ouabain (10^{-5} M)	91
Ouabain (10^{-4} M)	89
EGTA (0.5 mM)	96
EDTA (10 mM) + KCl (0.45 M)	0

[a] Oligomycin and ouabain inhibited H^+- and Na^+, K^+-ATPases, respectively, in our standard buffer as well as in ionic conditions optimal for these ATPases

The ATPase activity of the preparation in standard buffer was assumed to be 100% (this activity varied from 4.5–20.3 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$)

inhibiting H^+- and K^+, Na^+-ATPases [10,11], do not affect the ATPase activity of the tubulin preparation. Unlike the complete enzyme, the soluble fragment of H^+-ATPase is known to be insensitive to oligomycin, whereas it can be completely inactivated by short-term incubation in cold. At the same time, 30 min incubation of the tubulin preparation at 0°C results in no decrease in ATPase activity. Thus, it may be stated that the preparation is not contaminated with H^+- or K^+, Na^+-ATPases. The activity of the tubulin preparation was also not inhibited in the presence of 0.5 mM EGTA which shows that it is not due to the presence of Ca^{2+}-ATPase (table 2).

It is known that brain tissue contains significant amounts of actin and myosin. Myosin is an active ATPase, and actin splits ATP during polymerization. In this connection, it was important to elucidate whether the ATPase activity of the tubulin preparation was due to an actin or myosin admixture. We found that our ATPase completely loses its activity in the presence of 0.5 M KCl and 5 mM EDTA, whereas under the same conditions, myosin possesses ATPase activity [12]. We also demonstrated that actin obtained by us from rabbit skeletal muscles according to the method in [13] fails to split ATP under the conditions in which the activity was determined. Hence it can be said that the ATPase activity of the tubulin preparation is not due to the presence of actin or myosin.

4. Discussion

The results indicate that the preparation of bovine brain tubulin contains ATPase capable of cosedimenting with microtubules. The ATPase activity of this preparation is not due to the presence of contamination by known membrane ATPase or contractile proteins such as actin or myosin.

We cannot say as yet which of the polypeptides present in our preparation possesses ATPase activity. The only thing that can be stated definitely is that 6 S tubulin purified by gel filtration possesses no ATPase activity.

Although the specific ATPase activity of the tubulin preparation is low, it should be emphasized that the bulk of protein in our preparation is tubulin itself which is not ATPase, and the rest of proteins (including the ATPase) constitute not more than 1–2% each of the total mass of the preparation. In this connection, the specific activity of the ATPase studied seems to be at least 50 times higher than the experimentally recorded value, i.e., exceeds 0.5 $\mu mol \cdot mg^{-1} \cdot min^{-1}$.

The presence of ATPase activity in tubulin preparations was reported [14]. However, tubulin was precipitated with vinblastine which is not specific enough: in addition to tubulin, vinblastine is known to precipitate many other proteins [15]. Later, the presence of ATPase activity in cytoplasmic microtubule preparations was reported [16,17], but no inhibitor analyses were done in any of the cases. Moreover, a considerable part of ATPase activity was shown to be separated from microtubules or from tubulin by centrifugation or on gel filtration, respectively, and so the authors believed it to be due to contaminations [16].

We think it is possible that the ATPase studied here is inherent to cytoplasmic microtubules in vivo and ensures the mechanochemical coupling during their functioning.

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