Characterization of acid and alkaline phosphatase activity in preparations of tubulin and microtubule-associated proteins

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Acid and alkaline phosphatase activity, determined by the hydrolysis of *p*-nitrophenyl phosphate, was found in preparations of microtubules purified from bovine brain by temperature-dependent assembly–disassembly and ion-exchange chromatography. Phosphocellulose-purified tubulin contained an associated acid phosphatase activity, stimulated by Mg^{2+} and by Zn^{2+}. Alkaline phosphatase activity with a pH optimum of 10.4 was measured in a fraction of microtubule-associated proteins (MAPs). Kinetics and the effects of sodium fluoride, sodium tartrate, sulfhydryl-blocking agents, EDTA and Zn^{2+} are reported.

Tubulin *Microtubule-associated protein* *Acid phosphatase* *Alkaline phosphatase*

1. INTRODUCTION

Several important cellular functions depend on cytoplasmic microtubules, for example, mitosis, transport processes, secretion, and motility, but the factors regulating the assembly and disassembly of microtubules in vivo are not known. Reports of protein kinase activity associated with microtubule protein from a variety of sources (review [1]) raised the possibility that microtubule assembly and function are controlled by phosphorylation–dephosphorylation reactions mediated by cyclic nucleotides. However, while investigations have been centered upon microtubule-associated protein kinase activity, little is known about the associated phosphatase activity.

We have reported the presence of acid and alkaline phosphatase activity in preparations of microtubule protein from bovine brain [2]. This preparation consisted of ~80% tubulin and 20% microtubule-associated proteins (MAPs), and phosphatase activities at neutral pH were found in both fractions. However, it was not clear from this investigation whether acid phosphatase activity was an intrinsic property of tubulin and whether phosphatase activity of both the acid and alkaline types were to be found in the MAPs preparation. Here, we have addressed these questions and characterized the *p*-nitrophenylphosphatase activity associated with tubulin and MAPs after repeated cycles of assembly–disassembly, with respect to kinetic parameters and the effects of inhibitors.

2. MATERIALS AND METHODS

2.1. *Preparation of microtubule proteins*

Microtubule proteins were prepared from bovine brain by two cycles of temperature-dependent assembly–disassembly [3,4]. Tubulin was separated from MAPs by phosphocellulose ion-exchange chromatography [5]. The void volume consisted of tubulin, and the remaining proteins were eluted with 0.6 M NaCl (referred to here as 'unfractionated MAPs') or with 0.25 M NaCl (MAPs) in 20 mM Pipes (pH 6.8) and desalted on Sephadex G-25 equilibrated at 4°C with either 0.1 M citrate–HCl (pH 3.0, 4.0), Tris–HCl (pH 5.1, 5.5, 6.0, 6.8, 8.0) or glycine–NaOH (pH 9.1, 10.4, 11.5). Following chromatography, the protein fractions were used immediately for the experiments.

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2.2. *Phosphatase assay*

Acid and alkaline phosphatase activity was measured by the rate of hydrolysis of *p*-nitrophenylphosphate (*p*-Npp) at 37°C in the appropriate buffer [2]. The incubation medium consisted of proteins in the appropriate buffer and 5.5 mM *p*-Npp with or without 5 mM $MgSO_4$ or 0.5 mM $ZnSO_4$. Readings were made at 420 nm in a Zeiss spectrophotometer, using an extinction coefficient for *p*-nitrophenol of 14.330 $M^{-1}.cm^{-1}$. Maximum velocity (V) and Michaelis constants (K_M) were determined from Lineweaver-Burk plots by linear regression. Enzyme activity was expressed as nmol *p*-nitrophenol formed.mg $protein^{-1}.h^{-1}$. Protein concentration was determined according to [6] using bovine serum albumin as a standard.

2.3. *Electrophoresis*

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed using 4–15% gradient gels [7]. Gels were stained in 0.25% Coomassie brilliant blue in methanol:acetic acid:water (5:1:5, by vol.) and destained in 7% acetic acid and 5% methanol. Log M_r *vs* migration distance was plotted for the following protein standards (Bio-Rad) ($\times 10^{-3}$): myosin (200); β-galactosidase (116.25); phosphorylase B (92.5); bovine serum albumin (66.2); ovalbumin (45); carbonic anhydrase (31); soybean trypsin inhibitor (21.5); lysozyme (14.4). M_r-Values of microtubule proteins were then estimated from standard curves obtained by linear regression.

3. RESULTS AND DISCUSSION

Phosphocellulose-purified tubulin (fig. 1a) exhibited acid phosphatase activity at pH 5.1–6.8 (fig. 2a). At pH <5.1, tubulin precipitated. Addition of 5 mM Mg^{2+} stimulated the phosphatase activity, even at pH >8.0, where little or no activity

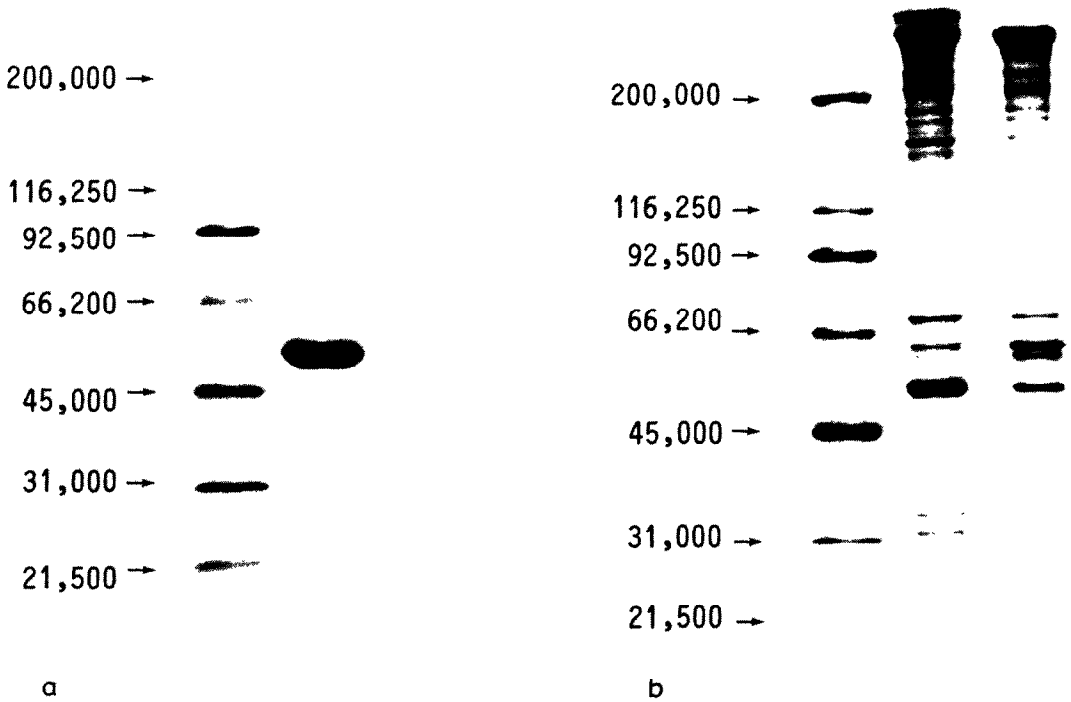

Fig. 1. SDS–polyacrylamide gradient (4–15%) gels of microtubule proteins: (a) lane 1, protein standards; lane 2, tubulin; (b) lane 1, protein standards; lane 2, unfractionated MAPs (single-step elution from phosphocellulose with 0.6 M NaCl) lane 3, MAPs (0.25 M NaCl phosphocellulose eluate).

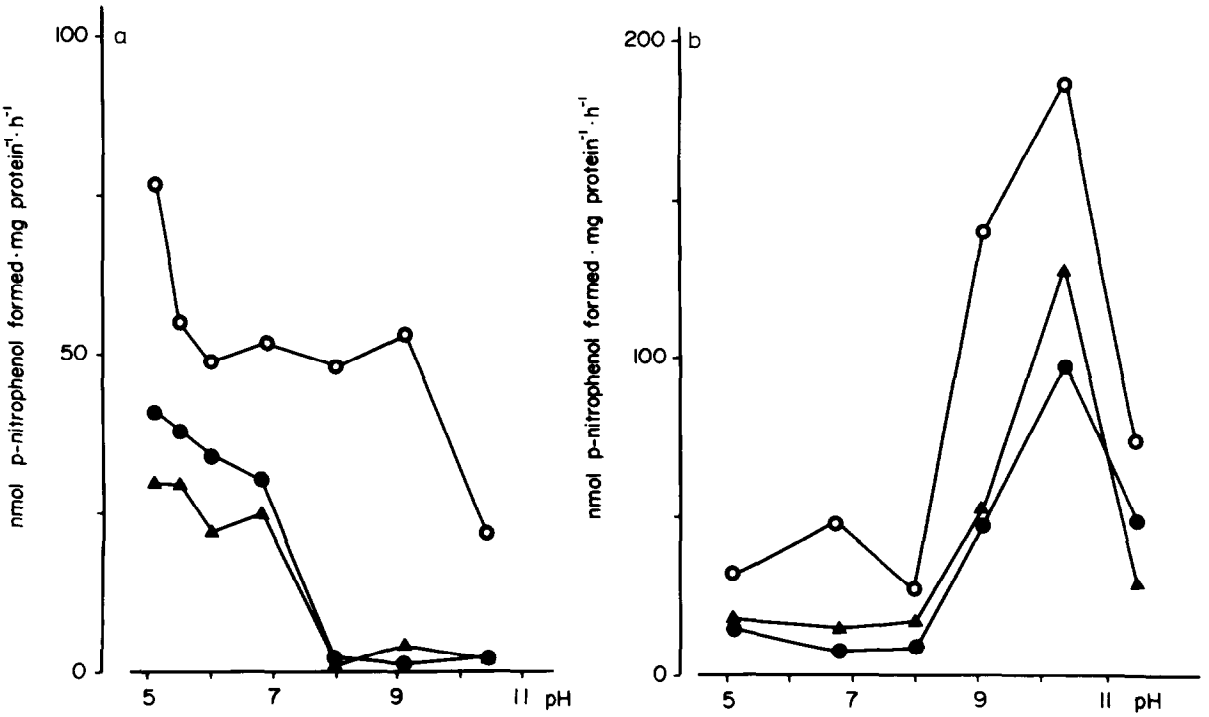

Fig. 2. pH dependence of (a) tubulin and (b) MAPs phosphatase activity. The incubation medium consisted of 0.5–1 mg tubulin/ml or 0.1–0.5 mg/ml MAPs in 0.1 M citrate–HCl (pH 3.0, 4.0), Tris–HCl (pH 5.1, 5.5, 6.0, 6.8, 8.0) or glycine-NaOH (pH 9.1, 10.4, 11.5), 5.5 mM *p*-Npp, in the absence of added metal ions (▲—▲), with 5 mM $MgSO_4$ (○—○) or 0.5 mM $ZnSO_4$ (●—●). Tubulin phosphatase activity was not determined at pH <5.1, as tubulin then precipitates.

Table 1

Effects of drugs on tubulin acid phosphatase activity (pH 5.1) and MAPs alkaline phosphatase activity (pH 10.4)

Drug	Tubulin phosphatase activity	MAPs phosphatase activity
pCMBS 0.1 mM	61%	95%
1.0 mM	23%	63%
NEM 10 mM	34%	86%
NaF 50 mM	45%	
+ 5 mM $MgSO_4$	25%	
Sodium tartrate 50 mM	41%	
+ 5mM $MgSO_4$	66%	
EDTA 0.0625–1 mM		7–0%
Zn^{2+} 0.001 mM		109%
0.01 mM		104%
0.5 mM		90%
10 mM		65%

Results are expressed as percent of control values

was found in the absence of added ions. A temperature optimum of 37°C was determined for the acid phosphatase activity (not shown). Activity was constant during 60 min and directly proportional to protein concentration within the range used for the experiments (0.5–1 mg/ml). Apparent K_M for *p*-Npp and V in the presence of 5 mM $MgSO_4$, calculated from Lineweaver-Burk plots by linear regression were 1.71 ± 0.27 mM and 1.74 ± 0.27 nmol *p*-nitrophenol formed.mg protein^{-1}.min^{-1}, respectively. The phosphatase activity was stimulated by 5 mM $MgSO_4$ and 0.5 mM $ZnSO_4$ (fig. 2a) and inhibited by high concentrations of pCMBS and NEM, sodium fluoride and sodium tartrate (table 1) as is the case for other acid phosphatases [8].

Acid phosphatase activity found in the unfractionated MAPs (fig. 1b) could be eluted with 0.6 M NaCl following the 0.25 M elution step (not shown), and may be accounted for by the presence of varying amounts of a M_r 57000 protein, possibly

tubulin, which co-elutes with this MAPs fraction. In contrast to tubulin, little phosphatase activity at acid pH was observed in the MAPs fraction (0.25 M eluate) (fig. 2b). This fraction consisted mainly of a high-M_r polypeptide (250000) in addition to small amounts of polypeptides of lower-M_r (50000–70000) (fig. 1b).

An alkaline phosphatase activity with a pH optimum around 10.4 (fig. 2b) and a temperature optimum of 37°C (not shown) was measured in the 0.25 M NaCl MAPs fraction. As was the case for tubulin, measurable amounts of phosphatase activity were also found over pH 5–8 upon Mg^{2+}-stimulation. This may be of significance for microtubule function, as the optimal pH for microtubule assembly, for example, is ~6.8. Alkaline phosphatase activity was constant for 60 min and directly proportional to the protein concentration used in the experiments (0.1–0.5 mg/ml). Mg^{2+} at 5 mM stimulated the activity and in its presence an app. K_M of 1.10 ± 0.14 mM and V of 1.75 ± 0.15 nmol p-nitrophenol formed.mg^{-1}.min^{-1} was determined.

SH-groups are apparently not involved in the activity of MAPs alkaline phosphatase activity, as only very high concentrations of pCMBS and NEM inhibit activity (table 1), a result which may be attributed to non-specific surface effects rather than to a specific interaction with thiols.

It has been suggested that Zn^{2+}-induced stimulation of tubulin phosphorylation [9] acts through the inhibition of a microtubule-associated phosphatase. However, 0.5 mM Zn^{2+}, which stimulated phosphorylation by 85%, inhibited the alkaline phosphatase activity by only 10% (table 1). Furthermore, low [Zn^{2+}] which inhibited phosphorylation by 70%, did not significantly stimulate phosphatase activity. This, however, does not exclude the possibility that Zn^{2+} inhibits a microtubule-associated phosphoprotein phosphatase.

EDTA was inhibitory at all concentrations tested (0.0625–0.1 mM) to ~7–0% of control values (table 1). This marked inhibition by EDTA may be an indication that the MAP phosphatase, like other alkaline phosphatases, is a metalloenzyme. Metal analysis of MAPs preparations, indicating traces of zinc and iron support this contention [10].

Some degree of caution should be exercised in interpreting the results of p-nitrophenylphosphatase assays of MAPs, as this fraction is known to contain an ATPase which hydrolyzes p-Npp as well as ATP, CTP, GTP and UTP [11]. However, p-Npp hydrolysis was very low compared to that of ATP (4%) and the kinetics and pH optimum of the ATPase differ from those of the phosphatase activity. It remains to be determined whether the p-nitrophenylphosphatase activity present in the 0.25 M NaCl eluate is specifically associated with microtubules in vitro and in vivo. However, preliminary results indicate that 65% of the total alkaline phosphatase activity in the original supernatant remains after 3 cycles of assembly–disassembly.

The main protein that is phosphorylated by a microtubule-associated protein kinase is MAP_2 [12], which has several phosphorylated sites/molecule [12,13], and may therefore require more than one enzyme for the phosphorylation or dephosphorylation of different sites [14]. During the course of microtubule-associated protein kinase assays, dephosphorylation upon increasing incubation times has been observed ([1,15,16], unpublished results), but no phosphoprotein phosphatase has been characterized or isolated. Some alkaline phosphatases are able to dephosphorylate phosphoproteins, in addition to low M_r substances like p-nitrophenol phosphate [14]. We are now investigating whether the phosphatases reported here have protein phosphatase activity and can thereby serve as a useful tool in investigating the role of the phosphorylated sites of MAPs.

Phosphocellulose-purified tubulin exhibited acid phosphatase activity (pH 5.1–6.8) and Mg^{2+}-stimulated phosphatase activity over pH 6.8–10.4; whereas, alkaline phosphatase activity with a pH optimum of 10.4 was found in a MAPs fraction containing proteins of M_r 250000 and 57000. Acid phosphatase activity is an intrinsic property of tubulin; the activity is inhibited by sodium fluoride and sodium tartrate. The MAPs alkaline phosphatase is likely to be a metalloenzyme, and the activity is associated with microtubules during at least 3 cycles of assembly–disassembly. The presence of non-specific acid and alkaline phosphatases in the microtubule system may provide a partial mechanism for microtubule function in secretory and transport processes and in interactions with membranes.

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