Microtubules and nucleoside diphosphate kinase

Nucleoside diphosphate kinase binds to co-purifying contaminants rather than to microtubule proteins

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Nucleoside diphosphate (NDP) kinase has been postulated to generate GTP from the GDP bound to tubulin. The purified chick brain enzyme was studied with respect to its kinetic parameters, and the protein–protein interactions between the NDP kinase and tubulin were examined. No specific interaction is observed between the enzyme and (i) assembled microtubules, (ii) tubulin dimers, or (iii) tubulin–microtubule-associated protein (MAP) oligomers under a variety of nucleotide conditions. The apparent association is demonstrated to result from NDP kinase binding to a co-purifying contaminant. The absence of detectable NDP kinase–tubulin interactions indicates that NDP kinase does not directly charge up tubulin–GDP.

INTRODUCTION

Microtubule assembly *in vitro* requires GTP or non-GTP nucleoside triphosphates such as ATP (Weisenberg, 1972). The present and the following paper (Islam & Burns, 1985) examine how non-GTP nucleoside triphosphates promote microtubule assembly.

GTP binds to two distinct sites on the tubulin dimer: an E-site, which is exchangeable with the medium, and a non-dissociating N-site. After addition of tubulin to the microtubule, the E-site GTP is hydrolysed (David-Pfeuty *et al.*, 1977), whereas the N-site remains unaltered (Penningroth *et al.*, 1976). The resultant E-site GDP remains bound and is non-exchangeable from the assembled microtubule (Weisenberg *et al.*, 1976).

Only GTP can exchange directly with the nucleotide bound to the tubulin dimer E-site: the K_d for ATP is about 1000 times that for GTP (Jacobs & Caplow, 1976). However, non-GTP nucleoside triphosphates support similar extents of microtubule assembly (Borisy *et al.*, 1974). The assembly induced by such triphosphates has been proposed to be mediated by NDP kinase generating GTP bound to the E-site by either (a) the direct charging of the tubulin:E-site GDP *in situ* (Penningroth & Kirschner, 1977; Huitorel *et al.*, 1984), or (b) the generation of GTP from free GDP as the substrate and the subsequent rebinding to the tubulin:E-site (Jacobs, 1979; Terry & Purich, 1979).

Direct charging of the E-site GDP has four inherent implications: (i) only GTP can bind to the E-site and promote microtubule assembly, (ii) the assembly effected by non-GTP triphosphates is mediated by the NDP kinase, (iii) the NDP kinase utilizes the E-site GDP and the non-GTP nucleoside triphosphates, and (iv) the NDP kinase interacts with tubulin.

The fourth implication, that there are significant protein–protein interactions between NDP kinase and microtubule protein, has not previously been examined. Such an interaction is indicated by the co-purification on

DEAE-chromatography (Zeeberg & Caplow, 1978; Berry & Shelanski, 1972), phosphocellulose chromatography (Penningroth & Kirschner, 1977; Jacobs, 1979), $(\text{NH}_4)_2\text{SO}_4$ precipitation and sucrose-density-gradient centrifugation (Berry & Shelanski, 1972). Furthermore, significant NDP kinase activity co-purifies through cycles of microtubule assembly/disassembly (Penningroth & Kirschner, 1977; Nickerson & Wells, 1978; Burns & Islam, 1981), indicating a reasonably strong protein–protein interaction.

In the present paper we examine whether chick brain NDP kinase is specifically associated with microtubule protein, but find no evidence for such protein–protein interactions. In the following paper, we examine the nucleotide requirements for microtubule assembly, and conclude that there is no evidence that NDP kinase is obligatory for the assembly by non-GTP triphosphates.

METHODS

Preparation of microtubule protein

Microtubule protein was purified through two cycles of assembly/disassembly from 1-day-old chick brains as previously described (Burns & Islam, 1981), except that the cold dissociation step was increased to 45 min for the first and to 2 h after the second cycle. The reassembly buffer consisted of 0.1 M-Mes, 2.5 mM-EGTA, 0.5 mM-MgCl₂, 0.1 mM-EDTA and 1 mM-dithiothreitol, adjusted to pH 6.4 with KOH. The microtubules consisted of tubulin and microtubule-associated protein 2 (MAP2) in stoichiometric amounts (Burns & Islam, 1982). When appropriate, 0.5–1.0 ml of the assembled microtubules were gently layered over a 5 cm-long sucrose cushion (6 ml; 30%, w/v, in reassembly buffer) and centrifuged at 120 000 g for 30 min at 30 °C.

NDP kinase

The chick brain soluble NDP kinase was purified and assayed as described previously (Islam & Burns, 1984a).

Abbreviations used: MAP, microtubule-associated protein; tubulin:E-site GTP or E-site GDP, tubulin with GTP or GDP bound to the exchangeable nucleotide-binding site; NDP kinase, nucleoside-diphosphate kinase (EC 2.7.4.6); once- and twice-cycle microtubule protein, protein purified through one or two cycles of assembly and disassembly.

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Table 1. Efficiency of nucleoside triphosphate utilization by the purified and microtubule-associated NDP kinase

NDP kinase activity was determined with 2.2 mM of various nucleoside triphosphates as the phosphate donor and 0.7 mM-TDP as the acceptor; the efficiencies are expressed as a percentage of the ATP-TDP exchange. Twice-cycled microtubule protein was assembled, pelleted through a 30% -sucrose cushion, cold-dissociated, clarified, and then assayed for NDP kinase activity.

Nucleoside triphosphate	Efficiency	(% of ATP-TDP exchange)
	Purified NDP kinase	3 × Microtubule protein
ATP	(100)	(100)
GTP	44	42
ITP	36	38
UTP	14	6*
CTP	3*	0*

*Measuring the very low activities observed using UTP and CTP as the phosphate donor is subject to considerable error.

One unit of NDP kinase activity is defined as the amount of enzyme that catalyses the reaction of 1 μ mol of ATP and 1 μ mol of TDP/min at 25 °C. The NDP kinase was stored in small batches in liquid nitrogen. Phosphorylated NDP kinase was prepared as described by Robinson *et al.* (1981).

Protein determination

Total protein concentrations were determined by the method of Hartree (1972), with bovine serum albumin as the calibration standard.

Nucleotide determination

Protein was adjusted to 5% (v/v) HClO_4 , incubated at 4 °C for 30 min, and then the precipitated protein was removed by centrifugation (18000 *g*, 15 min). The nucleotide concentration of the supernatant was determined by assuming an absorption coefficient, $\epsilon_{257}^{1\text{cm}}$ of 12.4×10^3 . Removal of unbound nucleotide and the partial emptying of the E-site GDP was achieved by treatment of the microtubule protein with activated charcoal (Penningroth & Kirschner, 1977).

Gel-exclusion chromatography

Protein was fractionated at 4 °C by gel-exclusion chromatography on a Bio-Gel A-1.5 m column (0.9 cm $\times$ 25 cm) pre-equilibrated with either reassembly buffer or, when appropriate, with phosphorylated NDP kinase.

Materials

All biochemical reagents were purchased from Sigma, except for pyruvate kinase (Calbiochem-Behring Corp.), lactate dehydrogenase (Boehringer) and Bio-Gel A-1.5 m (Bio-Rad Laboratories, Richmond, CA, U.S.A.). All other reagents were of Fisons A.R. grade.

RESULTS

Properties of chick brain NDP kinase

Chick brain NDP kinase is present in both the soluble and the particulate fractions (Islam & Burns, 1984a) whereas the bovine brain enzyme is primarily particulate (Robinson *et al.*, 1981). Only the chick soluble fraction (70–80% of total) is relevant to the current studies, as the same buffer and centrifugation conditions have been used to prepare the soluble NDP kinase and the microtubule protein. Preliminary studies indicate that the chick

soluble and particulate enzymes have common properties (Islam & Burns, 1984a).

The nucleotide specificity of the NDP kinase co-pelleted with assembled microtubules through a sucrose cushion is identical with that of the purified NDP kinase (Table 1), and is similar to that for purified bovine brain particulate NDP kinase (Robinson *et al.*, 1981) and for the soluble and microtubule-associated NDP kinase from bovine brain (Nickerson & Wells, 1984).

Double-reciprocal plots of the initial enzymic rate at fixed ATP concentrations and different concentrations of the TDP acceptor yield a family of parallel lines (Fig. 1), consistent with the 'Ping-Pong' bisubstrate mechanism. The corrected K_m for TDP (Fig. 1, insert) and K_m for

Fig 1. 'Ping-Pong' bisubstrate mechanism

NDP kinase activity was determined with ATP as the fixed substrate at 0.05 mM (○), 0.10 mM (▲), 0.30 mM (■) and 0.50 mM (●), and TDP as the changing substrate. Insert: the corrected K_m for TDP by reploting the data with TDP as the fixed substrate and ATP as the changing substrate was used to determine the $1/v$ intercepts at different TDP concentrations. The corrected K_m can be obtained by a plot of the $1/v$ intercepts versus the TDP concentrations and extrapolation to the abscissa intercept.

Fig. 2. Recovery of NDP kinase with assembled microtubules, as a function of the initial protein concentration

Increasing concentrations of twice-cycled microtubule protein were assembled with 1 mM-GTP in the absence (●,▲) and presence (○,△) of 5 units of purified NDP kinase for 30 min at 37 °C. The assembled microtubules were pelleted through a 30%-sucrose cushion, cold-dissociated, and assayed for protein (○,●) and NDP kinase activity (△,▲).

ATP, determined from the 1/velocity intercepts (Parks & Agarwal, 1973), are 9×10^{-4} M and 5×10^{-4} M respectively.

Association of NDP kinase with assembled microtubules

A sucrose cushion has been employed to separate the 'free NDP kinase' from the assembled microtubules, and has the additional advantage of preventing the recovery of tubulin-MAP2 oligomers (Burns & Islam, 1981). Increasing concentrations of twice-cycled microtubule protein (NDP kinase specific activity 0.25 unit/mg) was assembled with 1 mM-GTP with and without the addition of 5 units of purified NDP kinase. The assembled protein was pelleted through the sucrose cushion, and the recovery of protein and NDP kinase activity in the pellets was determined (Fig. 2). The recovery of the protein was directly proportional to the initial protein concentration and was not affected by the inclusion of NDP kinase. Similarly, the recovery of NDP kinase activity was also proportional to the initial concentration of microtubule protein. The inclusion of exogenous NDP kinase did not increase the specific activity (0.02 unit/mg) of the pelleted

microtubule protein, indicating that the added NDP kinase failed to bind to the assembled microtubules.

As the recovery of NDP kinase activity with the microtubule protein could be effected by the nucleotide conditions used to promote microtubule assembly, twice-cycled microtubule protein was charcoal-treated to remove excess nucleotides (residual of 1.6 mol of nucleotide/mol of tubulin) and then incubated with GTP, ATP, GDP, or GDP + ATP and the purified NDP kinase (5 units). The assembled microtubules were pelleted through a sucrose cushion and the NDP kinase activity of the pelleted protein was determined (Table 2). No significant differences in the total amount of NDP kinase activity was observed between the various conditions, except that no assembly occurred and consequently no pellets were recovered with GDP alone.

Association of NDP kinase with tubulin-MAP2 oligomers

Twice-cycled charcoal-treated microtubule protein was incubated with 3.5 units of purified NDP kinase and with 100 μ M-GDP, or 100 μ M-GDP + 1 mM-ATP, or 1 mM-ATP, and then fractionated on a Bio-Gel A-1.5 m column. The column fractions were assayed for NDP kinase activity and protein content (Fig. 3). Tubulin-MAP2 oligomers, representing 55–60% of the total protein, were eluted in the void volume and the tubulin dimers were eluted near the salt volume. By contrast, only a trace amount of NDP kinase was detected in the void fraction, with most of the enzymic activity being eluted just after the tubulin dimer peak, consistent with the M_r of the native NDP kinase (100 000) (Burns & Islam, 1981; Islam & Burns, 1984a). The observed distribution of the NDP kinase was unaffected by the nucleotide conditions.

Twice-cycled microtubule protein has also been eluted through a Bio-Gel A-1.5 m column pre-equilibrated with phosphorylated NDP kinase in a Hummel-Dreyer (1962)-type experiment. Pre-equilibration of the column with phosphorylated NDP kinase (0.35 unit/ml) resulted in a discrete void-volume peak (Fig. 4), with the specific activity increasing from 0.039 unit/mg in the absence of added NDP kinase to 0.204 unit/mg (after correction for the NDP kinase background), indicating substantial binding to material eluted in the void-volume fractions.

When once-cycled microtubule protein was fractionated in a similar experiment on the same column, there was also a marked increase in the specific activity of the void-volume fractions (Fig. 5). The mean specific activity

Table 2. Recovery of NDP kinase with microtubules through a sucrose cushion under various nucleotide conditions

Charcoal-treated twice-cycled microtubule protein was assembled under various nucleotide conditions in the presence of 5 units of purified NDP kinase. The assembled microtubules, pelleted through a sucrose cushion and cold-dissociated, were assayed for protein and NDP kinase activity.

Nucleotide conditions	Recovery of NDP kinase activity		
	Total activity (units)	Specific activity (units/mg)	(% of initial activity)
100 μ M-GTP	0.0098	0.023	0.18
1 mM-ATP	0.0088	0.041	0.16
1 mM-ATP + 100 μ M-GDP	0.0084	0.038	0.15
100 μ M-GDP	—	—	—

Fig. 3. Fractionation of charcoal-treated microtubule protein by gel-exclusion chromatography

Purified NDP kinase (3.5 units) was added to charcoal-treated twice-cycled microtubule protein in the presence of (a) 100 μ M-GDP, (b) 100 μ M-GDP + 1 mM-ATP and (c) 1 mM-ATP. The protein was then fractionated on a Bio-Gel A-1.5 m column pre-equilibrated in reassembly buffer, and eluted at 4 °C (flow rate 8 ml/h; 5 min per fraction). ●, Protein; ○, NDP kinase activity.

of 0.075 unit/mg in the absence of added NDP kinase increased to 0.556 unit/mg (corrected for NDP kinase background) when the column was pre-equilibrated with the enzyme.

Sodium dodecyl sulphate/polyacrylamide-gel electro-

phoresis of the void-volume material after elution of once- and twice-cycled microtubule protein showed that both consist primarily of MAP2 and tubulin. As similar amounts of protein were eluted in these fractions (once-cycled, 2 mg; twice-cycled, 2.75 mg), the number of tubulin oligomers must also have been roughly equivalent.

DISCUSSION

Chick brain NDP kinase, with an apparent pI of 8.1, has been purified to near homogeneity (Islam & Burns, 1984a), and has properties in common with NDP kinases isolated from various sources (Robinson *et al.*, 1981; Parks & Agarwal, 1973). It is hexameric with a subunit M_r of 16 500, contains essential thiol groups required for its activity, and functions via a 'Ping-Pong' mechanism (Fig. 1). Although it can use both purine and pyrimidine triphosphates as phosphoryl donors (Table 1), the utilization of the former is about 10 times that of the latter. This is in good agreement with that observed for the bovine brain NDP kinases (Robinson *et al.*, 1981; Nickerson & Wells, 1984).

Although the purified chick brain NDP kinase appears to be a unique species, some heterogeneity has been observed in crude extracts (Islam & Burns, 1984a). Such heterogeneity, similar to that reported for other tissues (Cheng *et al.*, 1971, 1973), raises the question of whether there is a specific tubulin-binding isoform. However, even mono-isoenzymic preparations of NDP kinase can yield multiple activity peaks, depending on the ionic conditions (Yue *et al.*, 1967), phosphorylation status (Colomb *et al.*, 1974), proteolytic degradation (Robinson *et al.*, 1981), and association with other proteins (Islam & Burns, 1984a). The purified chick brain NDP kinase exhibits the same nucleotide specificity as the activity that copellets with assembled microtubules (Table 1) and the particulate activity. Similarly, the NDP kinase associated with bovine (Nickerson & Wells, 1984) or pig (Huitorel *et al.*, 1984) brain microtubules have substrate specificities identical with those of the respective purified brain enzymes. It is therefore extremely unlikely that there is a specific tubulin-binding NDP kinase isoform.

If NDP kinase is able to utilize tubulin:E-siteGDP as a phosphoryl acceptor, then there must be an interaction between tubulin and the enzyme. Furthermore, as NDP kinase functions via a 'Ping-Pong' reaction (Fig. 1), this interaction must involve the phosphorylated intermediate, although binding of the unphosphorylated form is not precluded.

There is, however, no evidence for an interaction between the NDP kinase and tubulin dimers. The apparent M_r of the native NDP kinase hexamer is 100 000 (Islam & Burns, 1984a), so any tubulin dimer-NDP kinase complex should be eluted with an apparent M_r of 200 000 on gel-exclusion chromatography. For instance, the lactoperoxidase-tubulin complex can be detected by this method (Rousset & Wolffe, 1980). However, no such peaks of protein or NDP kinase activity were detected under a variety of conditions (Figs. 3 and 4), and NDP kinase fails to bind to a tubulin-Sepharose affinity column (Huitorel *et al.*, 1984).

However, the NDP kinase might be binding to tubulin-MAP2 oligomers, particularly as NDP kinase activity has been demonstrated to co-elute with an oligomeric species when once-cycled microtubule protein

Fig. 4. Binding of phosphorylated NDP kinase to microtubule protein examined by gel-exclusion chromatography

Twice-cycled microtubule protein was fractionated on a Bio-Gel A-1.5 m column which had been pre-equilibrated in reassembly buffer containing phosphorylated NDP kinase with an activity of 0.35 unit/ml. The column was eluted with the equilibration buffer (flow rate 5 ml/h) and the fractions were assayed for protein (A_{280} ; —) and NDP kinase activity (●). In addition to the major peak eluted in the void-volume fraction, a minor peak of NDP kinase activity (arrowed) is eluted shortly after the tubulin dimer, and corresponds to unbound NDP kinase hexamer.

was fractionated by gel-exclusion chromatography (Jacobs, 1979; Burns & Islam, 1981). However, little NDP kinase co-elutes with the oligomeric species when the more purified microtubule protein is fractionated (Fig. 3). Furthermore, adding purified NDP kinase, under a variety of nucleotide conditions, did not enhance the binding to the oligomeric species (Fig. 3).

The strongest evidence for an apparent interaction between NDP kinase and tubulin has come from the co-purification of the enzyme through successive cycles of microtubule assembly/disassembly (Penningroth & Kirschner, 1977; Nickerson & Wells, 1978; Burns & Islam, 1981). However, the specific activity of the NDP kinase co-pelleted with assembled microtubules is greatly decreased if a sucrose cushion is employed to minimize any entrapment (Fig. 2 and Table 2), or if the surface of the microtubule pellet is gently washed (Penningroth & Kirschner, 1977). Similarly, the addition of purified NDP kinase does not lead to an increased recovery of the enzyme with assembled microtubules, nor does it affect the final extent of assembly (Fig. 2). Finally, no enhanced binding was observed to microtubules challenged with NDP kinase in the presence of ATP, which will lead to the phosphorylation of the enzyme, and then pelleted through a sucrose cushion (Table 2).

There is consequently no evidence for a specific interaction of NDP kinase with microtubules, tubulin or MAP2-tubulin oligomers irrespective of the phosphorylation status. It is therefore necessary to consider the reason for the partial co-purification through cycles of assembly/disassembly and for the binding of phosphorylated NDP kinase to the material eluted in the Bio-Gel A-1.5 m void-volume fractions (Fig. 5).

Analysis of this binding of phosphorylated NDP kinase to material eluted in the void-volume on fractionation of

Fig. 5. Comparison of binding of phosphorylated NDP kinase to once- and twice-cycled microtubule protein Bio-Gel A-1.5 m void-volume fractions

Once- and twice-cycled microtubule protein was fractionated as described in Fig. 4. The distribution of (a) NDP kinase activity (once-cycled, ○; twice-cycled, ●) and (b) protein (A_{280} : once-cycled, ----; twice-cycled, —) in the void-volume fractions are compared.

once- and twice-cycled microtubule protein suggests that the enzyme binds to a high- M_r contaminant. The amount and protein composition of the void-volume fraction was similar for both preparations, so that the number of chargeable tubulin oligomers was roughly equivalent. However, once-cycled microtubule protein exhibited a higher specific activity in the absence of added NDP kinase and should therefore have shown a smaller increase in the specific activity if the NDP kinase binds to and saturates tubulin oligomers. This was not observed: the increase in the specific activity was 0.481 unit/mg for the once-cycled protein, but only 0.16 unit/mg for the twice-cycled protein. The recovery of the NDP kinase in the void-volume fraction is not consistent with binding to tubulin oligomers, indicating that the enzyme binds to a co-eluted contaminant which is selectively lost during cycles of microtubule assembly.

Warm supernatants after the second cycle of microtubule assembly, which contains little tubulin or MAP2,

show a peak of NDP kinase activity in the void-volume fractions when fractionated by gel-exclusion chromatography at 30 °C, but which was eluted near the salt volume when fractionated at 4 °C (K. Islam & R. G. Burns, unpublished work). This suggests that the NDP kinase-binding contaminant aggregates on warming, and such behaviour offers a ready explanation for the observed co-purification with microtubule protein. Similar behaviour has been observed for the co-purification of membrane-associated ATPase activity with microtubule protein (Murphy *et al.*, 1983). The findings with both the NDP kinase and the ATPase activity demonstrate the inherent difficulty in assigning functional roles to enzymic activities that co-purify with microtubule protein.

Any model for the promotion of microtubule assembly by non-GTP nucleoside triphosphates which involves the direct charging of tubulin:E-siteGDP by NDP kinase must involve an interaction between the NDP kinase and either tubulin dimers or the tubulin-MAP2 oligomers. However, the current experiments fail to detect any such protein-protein interactions. Comparison of the kinetics of microtubule assembly by GTP and non-GTP nucleoside triphosphates (Islam & Burns, 1984*b*, 1985) also shows that NDP kinase is not required for assembly, invalidating the apparent requirement for such protein-protein interactions. By contrast, challenging once- and twice-cycled microtubule protein with phosphorylated NDP kinase provides strong evidence that the NDP kinase binds to a minor contaminant present in preparations of microtubule protein.

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