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Nucleotide Interconversions in Microtubule Protein Preparations, a Significant Complication for Accurate Measurement of GTP Hydrolysis in the Presence of Adenosine 5′-(β,γ-Imidotriphosphate)

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ABSTRACT: Pursuing the observation of Carlier and Pantaloni [Carlier, M.-F., & Pantaloni, D. (1982) *Biochemistry 21*, 1215–1224] that adenosine 5′-(β,γ-imidotriphosphate) (pNHppA) strongly inhibited tubulin-independent phosphatases in microtubule protein preparations, we observed with a number of commercial preparations of pNHppA that a major proportion of the terminal phosphate of [γ-^{32}P]GTP added to microtubule protein preparations was rapidly converted into ATP. Initially postulating degradation of pNHppA to AMP followed by stepwise conversion of AMP to ATP, we isolated two nucleoside monophosphate kinase activities from microtubule protein capable of generating ATP from AMP + GTP. The amounts of these enzymes in microtubule protein preparations, however, are probably too low to account for rapid ATP formation. Instead, ATP formation most likely is caused by nucleoside diphosphate kinase acting on ADP contaminating commercial pNHppA preparations. Such ADP contamination was demonstrated by high-performance liquid chromatography, with the amount of ATP formed with different pNHppA preparations proportional to the amount of ADP contamination. Repurification of commercial pNHppA until it was free of contaminating ADP also resulted in the elimination of ATP formation. The repurified pNHppA potently inhibited GTP hydrolysis in microtubule protein preparations. In addition, especially when supplemented with equimolar Mg^{2+}, the repurified pNHppA strongly inhibited GTP hydrolysis and microtubule assembly in reaction mixtures containing purified tubulin and heat-treated microtubule-associated proteins (which contain negligible amounts of tubulin-independent phosphatase activity). We conclude that studies of microtubule-dependent GTP hydrolysis which make use of pNHppA must be interpreted with extreme caution.

Microtubule assembly generally requires GTP hydrolysis in amounts approximately stoichiometric with the amount of tubulin incorporated into polymer (MacNeal & Purich, 1978; Caplow & Zeeberg, 1980; Carlier & Pantaloni, 1982; Hamel et al., 1984). This GTP is located at the exchangeable nucleotide binding site of tubulin, for the equimolar amount of nonexchangeable GTP bound to the protein is unaltered during in vitro polymerization (Penningroth & Kirschner, 1977; Hamel et al., 1984) and may even persist without change in cellular microtubules (Spiegelman et al., 1977). Although many intriguing suggestions have been made to give GTP hydrolysis a more precise role in the assembly reaction or in the stabilization of microtubules [e.g., Kirschner (1980), Weisenberg (1980), Cote and Borisy (1981), Bonne and Pantaloni (1982), Carlier and Pantaloni (1982), Hill and Chen (1984), and Caplow and Reid (1985)], a severe limitation in the interpretation of data presented to support these ideas lies in the multiple enzyme contaminants found in microtubule protein preparations. These include nonspecific phosphatases which will degrade virtually any nucleotide, nucleoside diphosphate kinase, and adenylate kinase (Gaskin et al., 1974; Piras & Piras, 1977; Sutherland, 1976; Penningroth & Kirschner, 1977; Nickerson & Wells, 1978; Carlier & Pantaloni, 1982).

The phosphatases, in particular, by degrading GTP simultaneously and at a much faster rate than assembly-dependent GTP hydrolysis, obscure the reaction dependent on microtubule assembly. Carlier and Pantaloni (1982) demonstrated that the ATP analogue pNHppA,[1] supplemented by a molar equivalent of Mg^{2+}, markedly inhibited generation of P_i from GTP in preparations of microtubule protein. Assuming that the nonspecific phosphatases were virtually totally inhibited, that microtubule assembly and its associated GTP hydrolysis was unaffected by the high concentrations of either pNHppA or Mg^{2+} added to the reaction mixture, and that no

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[1] Abbreviations: pNHppA, adenosine 5′-(β,γ-imidotriphosphate); MAP(s), microtubule-associated protein(s) (for the purposes of this study, a MAP is defined as any protein other than tubulin present in two-cycle microtubule protein preparations); Mes, 4-morpholineethanesulfonate; PEI–cellulose, poly(ethylenimine)–cellulose; TEAB, triethylammonium bicarbonate; NH_2ppA, adenylyl phosphoramidate; Pipes, 1,4-piperazinediethanesulfonate; P_i, inorganic phosphate.

other reactions of significance occurred when pNHppA was added to reaction mixtures, Carlier and Pantaloni (1982) analyzed in great detail the amount of P_i formed relative to the extent of microtubule assembly as measured by turbidimetry and centrifugation.

Our initial interest in pNHppA was stimulated by this report of Carlier and Pantaloni (1982), for we thought that the analogue could be employed as a tool for the development of an enzymatic assay for MAPs to use in the purification of these proteins. We attempted to define specific activity of MAP preparations by the amount of tubulin-dependent GTP hydrolysis that occurred in the presence of pNHppA. In our laboratory, however, we use a thin-layer chromatography/autoradiography technique to measure GTP hydrolysis (in which an aliquot of the entire reaction mixture is analyzed) rather than a P_i extraction method (Carlier & Pantaloni, 1982). Therefore, multiple reactants and/or products can be visualized, depending on the specific radiolabeled compounds and enzymes in the reaction mixture. When we examined the fate of [γ-^{32}P]GTP in microtubule protein preparations supplemented with pNHppA, we, too, found a marked reduction in the amount of P_i formed. In addition, however, we found that a substantial amount of radiolabeled phosphate from [γ-^{32}P]GTP was diverted into a new nucleotide component that was not formed in the absence of pNHppA. This report describes the identification of this component, our efforts to determine how it arises in the microtubule protein preparation upon addition of the ATP analogue, and our initial evaluation of whether pNHppA $\pm$ Mg^{2+} is indeed an innocent addition to a microtubule assembly reaction mixture.

Materials and Methods

Materials. Microtubule protein, purified tubulin, and heat-treated MAPs were prepared as described elsewhere (Hamel & Lin, 1984). The following nucleotides (nonradiolabeled obtained from Sigma, radiolabeled from Moravek) were repurified by triethylammonium bicarbonate gradient elution from DEAE-Sephadex A-25: GTP, GDP, ATP, CTP, UTP, [8-^{14}C]GMP, [8-^{14}C]GDP, and [8-^{14}C]GTP. The following nucleotides (nonradiolabeled obtained from Sigma, radiolabeled from Amersham) were used without further purification: AMP, ADP, GMP, CMP, UMP, [8-^{14}C]AMP, [U-^{14}C]ADP, and [U-^{14}C]ATP. The method of Walseth and Johnson (1979) was used to prepare [γ-^{32}P]GTP, and the nucleotide was purified by triethylammonium bicarbonate gradient elution from DEAE-Sephadex A-25. PEI–cellulose thin-layer sheets were obtained from Brinkmann.

Purification of pNHppA and NH_2ppA. One or more samples of pNHppA were obtained from four different commercial sources. All were found to contain significant amounts of NH_2ppA by thin-layer chromatography. Commercial preparations were therefore purified by TEAB gradient elution from DEAE-Sephadex A-25. NH_2ppA was obtained as a byproduct. Column size was 2.5 × 50 cm, development was with a 1.9-L gradient from 0 to 1.0 M TEAB (pH 8.2), and the pNHppA and NH_2ppA peaks were at approximately 0.75 and 0.4 M TEAB, respectively. The peak fractions of pNHppA, containing approximately 75% of the analogue, were pooled, diluted, reapplied to a DEAE-Sephadex A-25 column, and reeluted with a TEAB gradient. Since previous qualitative studies had indicated that the triethylammonium salt of pNHppA was not stable, half the purified pNHppA was converted to the lithium salt by precipitation in ethanol with LiCl. The two salts were stored at both −20 °C and in liquid nitrogen for about 8 months and then analyzed by high-performance liquid chromatography (see below). The lithium salt was stable at both temperatures, while the triethylammonium salt was stable only in liquid nitrogen (at −20 °C about 10% had broken down to NH_2ppA, with no other degradation products detected).

Partial Purification of Nucleoside Diphosphate Kinase, AMP Kinase, and GMP Kinase from Microtubule Protein. Beginning with 19.3 g of microtubule protein, we prepared a MAP-enriched supernatant fraction as described previously (Hamel & Lin, 1984). This contained the three enzymatic activities, and the purification of nucleoside diphosphate kinase has been described elsewhere (Hamel et al., 1986). Both the AMP kinase and GMP kinase are active with AMP as phosphate acceptor and GTP as donor (see below), and until their resolution on hydroxyapatite, the enzymatic activity was followed by generation of [8-^{14}C]ADP and [8-^{14}C]ATP from [8-^{14}C]AMP in the presence of nonradiolabeled GTP. After their differential nucleotide specificities were noted, aliquots from the different steps in the purification procedure were reassayed with ATP as phosphate donor for both enzymes but [8-^{14}C]AMP as acceptor with AMP kinase and [8-^{14}C]GMP as acceptor with GMP kinase. Activities were quantitated in international units (IU).

The MAP-enriched supernatant fraction (2.0 g of protein containing 162 IU of AMP kinase and 8.1 IU of GMP kinase) was applied to a 5 × 70 cm column of DEAE-Sephacel (from Pharmacia) as described before (Hamel & Lin, 1984). The protein (410 mg) not retained by the column contained most of the AMP and GMP kinase activities, and they were precipitated with $(NH_2)_2SO_4$ (60–100% fraction, 120 mg of protein). This protein was applied to a preparative TSK G3000SWG high-performance liquid chromatography column (2.15 × 60 cm), and the two enzymes coeluted in a peak containing 51 mg of protein. The protein was applied to a 1.5 × 5.0 cm column of hydroxyapatite (Bio-Gel HT, from Bio-Rad). The nucleoside monophosphate kinase activity, as assayed with the AMP/GTP combination, was recovered as two distinct peaks. The first (7.9 mg of protein, subsequently identified as GMP kinase) did not bind to hydroxyapatite, while the second activity peak (8.4 mg of protein, subsequently identified as AMP kinase) was in the first protein eluted by the phosphate gradient. Overall purification from the soluble MAP fraction of the AMP kinase was about 84-fold (yield 36%) and that of the GMP kinase about 57-fold (yield 22%). The final preparations contained multiple bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with specific activities of 6.9 IU/mg for AMP kinase and 0.23 IU/mg for GMP kinase.

Thin-Layer Chromatography and Autoradiography. In general, a 5- or 10-μL aliquot of a reaction mixture was added to twice as much 25% acetic acid, which contained appropriate nucleotide standards, to stop the reaction. Ten microliters of the mixture was then spotted on either a half or a full plastic-backed PEI–cellulose thin-layer sheet, and chromatography was begun in 0.5 M KH_2PO_4 (pH unadjusted) while the spots were still wet. After ascending chromatography, the PEI–cellulose sheet was thoroughly dried, marked with radioactive ink, and exposed to X-ray film (generally for less than 1 h for ^{32}P-labeled nucleotides, for 12–24 h for ^{14}C-labeled nucleotides). The autoradiogram was overlaid with the chromatogram on a light box, the chromatogram was marked, and appropriate areas were cut from it for quantitation of nucleotide hydrolysis or interconversions.

High-Performance Liquid Chromatography. Different preparations of pNHppA were analyzed on a Whatman Partisil SAX column (0.46 × 25 cm), by use of an LKB

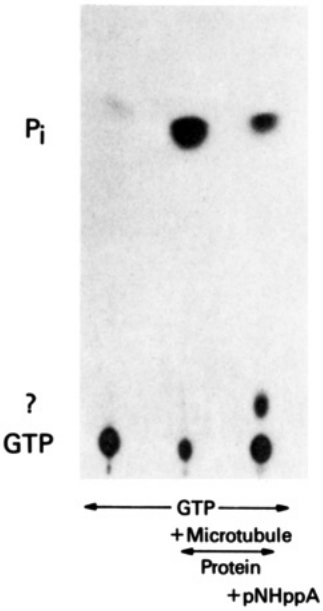

FIGURE 1: Formation of a new nucleotide by microtubule protein in the presence of [γ-^{32}P]GTP and commercial pNHppA. Each 50-μL reaction mixture contained 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]GTP, and, as indicated, 1.5 mg/mL microtubule protein and 1.0 mM pNHppA. Incubation was for 10 min at 37 °C. Aliquots from the samples were processed as described in the text, with about 100 000 cpm spotted from each sample. The radiopurity of the [γ-^{32}P]GTP used in this experiment was 95%. Quantitative analysis of the chromatograms was as follows: in the sample with microtubule protein only, 81.2 nmol/mL of P_i was formed; in the sample with pNHppA, 12.5 nmol/mL of the new nucleotide and 15.8 nmol/mL of P_i were formed. (Thus, about one-eighth of the γ-phosphate of GTP was diverted into the new nucleotide, and overall hydrolysis was reduced about 80%.)

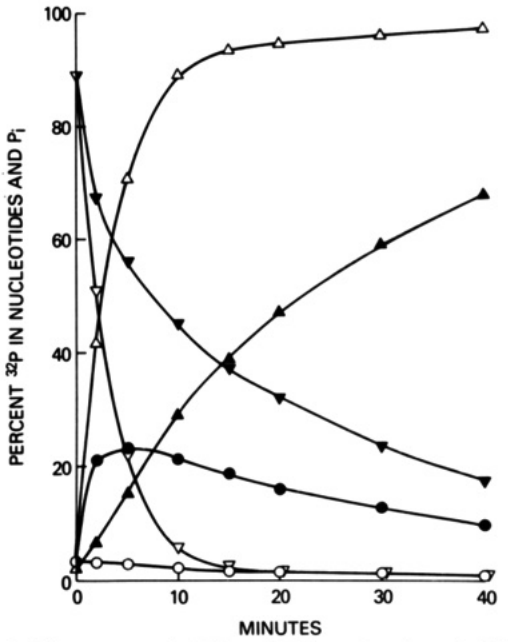

FIGURE 2: Time course of ATP and P_i formation from [γ-^{32}P]GTP by microtubule protein in the presence of commercial pNHppA. Each 50-μL reaction mixture contained 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]GTP, 1.5 mg/mL microtubule protein, and no further addition (open symbols) or 1.0 mM pNHppA (closed symbols) (zero time values were obtained from reaction mixtures containing all components except microtubule protein). At the indicated times at 37 °C, 5-μL aliquots were removed from the reaction mixtures and processed as described in the text. The chromatogram of each sample was cut into portions corresponding to the regions at which GTP, ATP and P_i migrated, and these were counted (all other portions of each track were combined into a fourth sample for counting). Data are therefore expressed as the percent of the total counts per minute (cpm) migrating as GTP (▽, ▼), ATP (○, ●), and P_i (△, ▲). In this experiment, the radiopurity of the [γ-^{32}P]GTP was 89%, and about 350 000 cpm was spotted from each sample.

high-performance liquid chromatography system equipped with a recording integrator. Two different gradients were used. In both cases the nucleotides were bound to the column in water. They were then eluted, at a flow rate of 2.5 mL/min, with a linear gradient either over 30 min to 0.6 M ammonium phosphate (pH 3.8) or over 20 min to 0.3 M ammonium phosphate (pH 6.5). The former gradient resulted in a wider separation of AMP and NH_2ppA (with AMP eluting first) but no separation of ATP and pNHppA. The latter gradient (see below) produced good separation of ATP and pNHppA but a narrow separation of AMP and NH_2ppA (with NH_2ppA eluting first). There was no significant difference in the quantitation of contaminants (except for ATP) in any preparation with the two different gradients.

Microtubule Assembly. This was evaluated by turbidimetry (Gaskin et al., 1974) at 350 nm by using a Gilford Model 250 recording spectrophotometer equipped with an electronic temperature controller. At zero time the controller was set at the indicated temperature, and the temperature of the reaction mixtures in the cuvettes rose at a rate of approximately 0.5 °C/s.

Results and Discussion

The Problem. Carlier and Pantaloni (1982) reported that pNHppA (with equimolar Mg^{2+}) markedly inhibited the generation of $^{32}P[P_i]$ from [γ-^{32}P]GTP by microtubule protein preparations. These workers, however, limited their analysis of reactants and products to an extraction of phosphate from reaction mixtures. When we analyzed an aliquot of a comparable reaction mixture by thin-layer chromatography/autoradiography (Figure 1), we found that the reaction was more complex than Carlier and Pantaloni (1982) reported. While there was indeed an obvious reduction in the amount of P_i formed in the presence of pNHppA, a significant proportion of the radiolabeled phosphate appeared in a new spot.[2] Two successive preparations of pNHppA from the same supplier resulted in substantial diversions of the radiolabeled phosphorus into this new compound.

Our first priority was identification of this unexpected nucleotide, for formation of radiolabeled pNHppA, in particular, would have been extremely interesting. Using appropriate nucleotide markers and various chromatographic conditions, however, we established that the newly formed material was ATP (data not presented).[3]

We next examined the time course of formation of ATP in relation to the degradation of GTP in a microtubule protein preparation supplemented with pHNppA (Figure 2). While GTP hydrolysis continued over the entire time period examined, the amount of ATP formed reached maximum levels within 5–10 min and then declined slowly. Figure 2 also

[2] The amount of this new nucleotide was not greatly affected by whether or not an additional 1.0 mM $MgCl_2$ was included in the reaction mixture. In most cases the supplementary Mg^{2+} was not added to the reactions.

[3] The maximum separation of ATP (R_f = 0.33) and pNHppA (R_f = 0.47) was observed in an experiment performed as follows: The reaction mixture was chilled to 0 °C after 5 min at 37 °C and diluted with 2 parts cold water. Aliquots were spotted directly on PEI–cellulose and overspotted with either ATP or pNHppA standards. Chromatography was begun in 1.5 M LiCl while the spots were still wet. When the chromatogram was completely developed, it was dried and the standards were visualized under ultraviolet light. An autoradiogram was prepared and compared to the chromatogram. Radioactivity coincided only with the position of the ATP standard. The pNHppA region was clear.

Table I: Nucleotide Specificity of Nucleoside Monophosphate Kinase Activities Separated on Hydroxyapatite[a]

	Experiment I: Radiolabeled Acceptors			
	[8-^{14}C]AMP[b] (nmol of product formed)		[8-^{14}C]GMP[c] (nmol of product formed)	
triphosphate donor	AMP kinase	GMP kinase	AMP kinase	GMP kinase
ATP	205	3	0	224
GTP	68	131	0	84
CTP	119	0	0	92
UTP	79	0	0	34
	Experiment II: Radiolabeled Donors			
	[U-^{14}C]ATP[d] (nmol of product formed)		[8-^{14}C]GTP[e] (nmol of product formed)	
mono-phosphate acceptor	AMP kinase	GMP kinase	AMP kinase	GMP kinase
AMP	205	5	88	167
GMP	0	182	0	83
CMP	121	0	6	1
UMP	136	0	101	1

[a] Each 40-μL reaction mixture contained 0.1 M Pipes (adjusted to pH 7.0 with NaOH), 0.5 mM $MgCl_2$, 1.0 mg/mL bovine serum albumin, the indicated nucleoside monophosphate at 0.25 mM, the indicated nucleoside triphosphate at 0.5 mM, and either 10 μg/mL GMP kinase or 16 μg/mL AMP kinase (the unbound and bound nucleoside monophosphate kinase peaks, respectively, from the final hydroxyapatite step in the purification procedure). Incubation was for 30 min at 37 °C. Aliquots of each reaction mixture were analyzed by thin-layer chromatography on PEI–cellulose and autoradiography, with product formation expressed as nmol/mL of reaction mixture. [b] The amounts of [8-^{14}C]ADP and [8-^{14}C]ATP formed were determined and summed. [c] The amounts of [8-^{14}C]GDP and [8-^{14}C]GTP formed were determined and summed. [d] The amounts of [U-^{14}C]AMP and [U-^{14}C]-ADP formed were determined and summed. [e] The amounts of [8-^{14}C]GMP and [8-^{14}C]GDP formed were determined and summed.

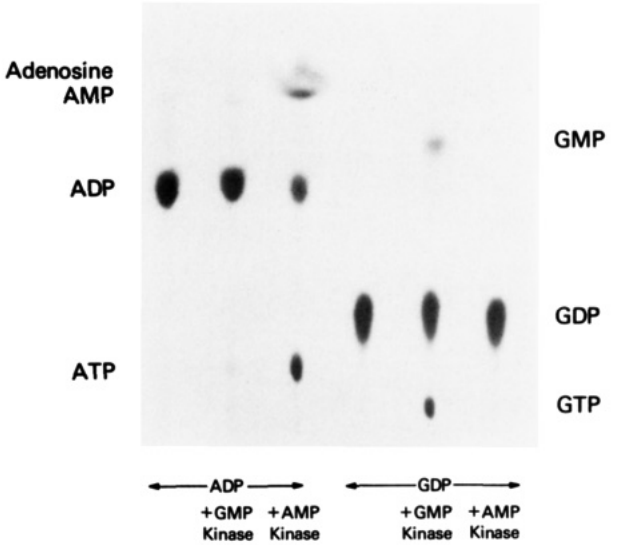

FIGURE 3: Differing nucleotide specificities of AMP kinase and GMP kinase. Each 50-μL reaction mixture contained 0.1 M Pipes (pH 7.0), 1.0 mM $MgCl_2$, 1.0 mg/mL bovine serum albumin, 100 international milliunits (ImU)/mL GMP kinase or AMP kinase, as indicated, and either 0.5 mM [8-^{14}C]GDP or 0.5 mM [U-^{14}C]ADP, as indicated. After 15 min at 37 °C, an aliquot of each sample was processed as described in the text, with about 130 000 cpm spotted from each sample. The radiopurities of the [8-^{14}C]GDP and [U-^{14}C]ADP used in this experiment were 97% and 90%, respectively. Quantitative analysis of the chromatograms (adenosine and AMP were combined for quantitation as AMP) was as follows: in the sample with [U-^{14}C]ADP + GMP kinase, 7 nmol/mL AMP and 5 nmol/mL ATP were formed; with [U-^{14}C]ADP + AMP kinase, 160 nmol/mL AMP and 166 nmol/mL ATP were formed; with [8-^{14}C]GDP + GMP kinase, 61 nmol/mL GMP and 80 nmol/mL GTP were formed; with [8-^{14}C]GDP + AMP kinase, 0.3 nmol/mL GMP and 0.1 nmol/mL GTP were formed.

demonstrates that the initial rate of P_i formation was inhibited approximately 90% by the pNHppA preparation used in this experiment.

Finally, in our initial studies, we had attempted to develop a method to quantitate MAP specific activity on the basis of tubulin-dependent GTP hydrolysis in the presence of pNHppA, examining protein preparations at various stages in a purification scheme that has been described in detail elsewhere (Hamel & Lin, 1984). Autoradiograms of reaction mixtures containing MAPs, pNHppA, and [γ-^{32}P]GTP demonstrated substantial ATP formation in the total soluble MAPs fraction and in the MAPs fraction that did not bind to DEAE-cellulose (flow-through MAPs), but not in the MAPs fraction eluted from DEAE-cellulose with NaCl [the latter contains most of the τ factor, MAP-1 and MAP-2 of the microtubule protein—see Hamel and Lin (1984)]. We therefore concentrated our attention on the flow-through MAPs.

The Red Herring: Nucleoside Monophosphate Kinase. One route to ATP, starting with pNHppA and GTP, would be a one-step breakdown of the analogue to AMP with subsequent phosphate transfers to the monophosphate. Although this would require several enzymes, the key initial reaction, enzymatic breakdown of pNHppA to AMP, has been well documented (Yount et al., 1971). Moreover, we found that adding nonradiolabeled GTP and [8-^{14}C]AMP to the flow-through MAPs resulted in significant ATP formation. In purifying this enzyme, we obtained two distinct fractions after hydroxyapatite chromatography with major differences in substrate specificity (Table I; Figure 3). Most notably, the hydroxyapatite-bound enzyme was unable to phosphorylate GMP, while the activity not bound by hydroxyapatite phosphorylated GMP with all ribotriphosphates, but AMP only with GTP as phosphate donor (Table I). In addition, only the hydroxyapatite-bound activity converts ADP to a mixture of AMP, ADP, and ATP,[4] while only the activity not bound to hydroxyapatite converts GDP to a mixture of GMP, GDP, and GTP (Figure 3). The former activity probably represents the AMP kinase described by other workers as a component of microtubule protein preparations (Gaskin et al., 1974; Sutherland, 1976), while the latter is another nucleoside monophosphate kinase, previously undescribed in microtubule protein, best termed GMP kinase.

At this stage we again examined both components of the nucleoside monophosphate kinase activity with pNHppA and [γ-^{32}P]GTP, anticipating that no reaction would occur. To our surprise, there was still substantial radiolabeled ATP formation, and properties of the reactions with the two enzymes were examined in further detail. These studies led us to conclude that neither nucleoside monophosphate kinase activity was likely to be responsible for most of the ATP formation in microtubule protein when pNHppA and GTP were added to the reaction mixture. Two considerations led us to this conclusion.

First, the formation of ATP with both enzymes was relatively slow. Even when amounts of AMP kinase and GMP kinase (equivalent to about 10 times that estimated to be

[4] Note that a residual phosphatase contaminates the AMP kinase preparation. This may be specific for AMP, for in other experiments there was no degradation of ATP or GTP (data not presented); and in the experiment of Figure 3 no degradation of GDP occurred. The chromatography in the experiment of Figure 3 was performed on a full sheet of PEI–cellulose, while that of the experiment of Table I was performed on a half-sheet of PEI–cellulose. In the latter case, the resolution of AMP and adenosine was not apparent.

Table II: Formation of ATP from [γ-^{32}P]GTP and Commercial pNHppA by AMP Kinase, GMP Kinase, and Nucleoside Diphosphate Kinase[a]

	ATP formed (nmol/mL)	
	10 min	60 min
experiment I		
+AMP kinase (10×)	0.7	5.4
+GMP kinase (10×)	0.8	10.3
experiment II		
+NDK[b] (10×)	34.0	33.1
+NDK (1×)	25.3	33.5
+NDK (0.1×)	7.0	21.5

[a] Each 50-μL reaction mixture contained 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]GTP, 1.0 mM pNHppA, 0.5 mg/mL bovine serum albumin, and one of the following enzymes, in an amount equivalent to the multiple of that present in the MAP-enriched supernatant fraction: 130 international milliunits (ImU)/mL AMP kinase, 6.3 ImU/mL GMP kinase, or 170, 17, or 1.7 ImU/mL nucleoside diphosphate kinase. Incubation was for the indicated times at 37 °C. Reactions were evaluated by thin-layer chromatography/autoradiography as described in the text. [b] NDK, nucleoside diphosphate kinase.

present in two-cycle microtubule protein), separately (Table II, experiment I) or in combination (data not presented), were mixed with the nucleotides, little ATP was formed at short incubation times. The ATP accumulated over a long period of time, a pattern quite distinct from that observed with microtubule protein (Figure 2). Second, there was no significant nonspecific triphosphatase activity in either the AMP kinase or GMP kinase preparation, nor did either preparation degrade radiolabeled pNHppA (data not presented).

The Accomplice: Nucleoside Diphosphate Kinase. In studies presented previously (Hamel & Lin, 1984) we had demonstrated that most of the soluble nucleoside diphosphate kinase associated with microtubule protein was recovered in the flow-through MAPs. This enzyme was purified in tandem with the nucleoside monophosphate kinases [described in detail in Hamel et al. (1986)]. Although it was not obvious how ATP could be generated from pNHppA (assuming the latter is nonhydrolyzable), we mixed the analogue and [γ-^{32}P]GTP with the partially purified nucleoside diphosphate kinase. To our surprise, ATP was rapidly formed (Table II, experiment II), with little apparent difference from the reaction originally observed in microtubule protein. A significant reaction was observed even with the equivalent of only 10% of the enzyme activity present in the total soluble MAPs. Since the nucleoside diphosphate kinase preparation contained little, if any, phosphatase or nucleoside monophosphate kinase activities and did not degrade radiolabeled pNHppA (data not presented), it was difficult to visualize how a presumably multistep generation of ATP from pNHppA and GTP could occur.

The Culprit: Contaminants in Commercial pNHppA. Examination of numerous commercial preparations of pNHppA by thin-layer chromatography on PEI–cellulose (usually 10–25 nmol were spotted) revealed universal contamination with NH_2ppA, but there was no obvious AMP or ADP in these preparations. Nevertheless, when commercial pNHppA was repurified by ion-exchange chromatography and the repurified analogue added to microtubule protein along with [γ-^{32}P]GTP, no ATP was formed (Figure 4). Moreover, inhibition of P_i formation was substantially greater with repurified pNHppA than with the commercial preparation used in this study (only about 25% as much P_i was formed when the repurified pNHppA was present as compared to the commercial preparation).

A number of commercial preparations of pNHppA were therefore examined by high-performance liquid chromatography. Multiple contaminants were observed in some of these preparations, and Figure 5 presents the profile of one of the most contaminated preparations (Figure 5B), one of the least contaminated (Figure 5C), and the repurified pNHppA (Figure 5D).[5] (Figure 5A presents the profile obtained with approximately equimolar concentrations of AMP, ADP, ATP, NH_2ppA, and pNHppA.)

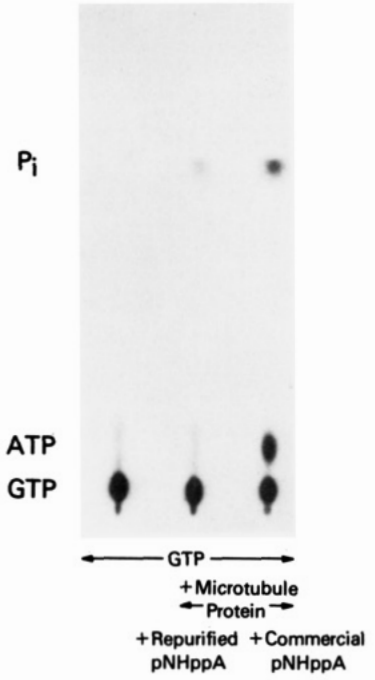

FIGURE 4: ATP is not formed by microtubule protein with repurified pNHppA. Each 50-μL reaction mixture contained 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]GTP, and as indicated, 1.5 mg/mL microtubule protein and 1.0 mM commercial pNHppA or 1.0 mM repurified pNHppA. Incubation was for 5 min at 37 °C. Aliquots from the samples were processed as described in the text, with about 450 000 cpm spotted from each sample. The radiopurity of the [γ-^{32}P]GTP used in this experiment was 92%. Quantitative analysis of the chromatograms was as follows: in the sample with repurified pNHppA, 2.6 nmol/mL P_i but no ATP was formed: in the sample with commercial pNHppA, 9.8 nmol/mL P_i and 21.8 nmol/mL ATP were formed. (In a control sample run simultaneously with microtubule protein but no pNHppA, 67.8 nmol/mL P_i was formed.)

Additional details are presented in Table III, in which results of high-performance liquid chromatographic analysis of seven

[5] Upon obtaining these results, we reevaluated the potential of thin-layer chromatography for detecting small amounts of ADP in commercial pNHppA preparations. We have obtained the best separation of the two nucleotides on PEI–cellulose in 0.5 M KH_2PO_4 (pH unadjusted), with R_f values of 0.19 and 0.34 for pNHppA and ADP, respectively. Solutions containing defined amounts of ADP and pNHppA were prepared, 100 nmol of total nucleotide from each solution was spotted on a half-sheet of PEI-cellulose, and the plate was developed in 0.5 M KH_2PO_4. With this amount of nucleotide the ADP and pNHppA spots merged, and the lower limit of detectability for ADP was approximately 5% ADP + 95% pNHppA. At the same time, however, the lower limit of detectability for a nucleotide with minimal contamination is about 0.5–1.0 nmol. Thus, limits for detecting impurities vary with the relative amounts and mobilities of nucleotides in a given preparation. With the information provided by high-performance liquid chromatography, commercial preparation 5 of pNHppA was examined again. When 100 nmol of total nucleotide was spotted, ADP and ATP were easily visible, largely because so little pNHppA was present. The KH_2PO_4 solution, however, does not resolve NH_2ppA and AMP, while 1.5 M LiCl does. Even using this latter solvent, we could not detect AMP in pNHppA preparation 5. We therefore conclude that high-performance liquid chromatography is substantially more sensitive than thin-layer chromatography for evaluation of nucleotides for contaminants.

Table III: Analysis of the Purity of Various Preparations of pNHppA by High-Performance Liquid Chromatography and Correlation with Effects on ATP and P_i Formation from [γ-^{32}P]GTP by Microtubule Protein

pNHppA preparation[a]	nucleotides present[b] (%)					formation by microtubule protein[c] (nmol/mL)	
	pNHppA	ADP	AMP	ATP	NH_2ppA	ATP	P_i
1 (supplier A)	30	3.6	5.7	2.7	53	22	21
2 (supplier A)	61	0.6	1.9	0.4	34	5.7	7.2
3 (supplier A)	80	0.6	1.0	0.3	17	5.2	6.1
4 (supplier A)	89	0.1	0.5	trace	9.9	1.3	4.0
5 (supplier B)	29	3.9	2.1	4.3	48	25	22
6 (supplier C)	89	trace	trace	0	11	0.5	4.5
7 (supplier D)	89	0	0.4	0	11	0.5	6.1
repurified	99	0	0.5	0	0.2	0	4.2

[a] All preparations except preparation 7 were the lithium salts. Preparation 7 was supplied as the sodium salt. [b] Details of the high-performance liquid chromatography are provided in the text. The analysis of each pNHppA preparation was performed on 0.3 μmol of nucleotide. [c] Each 50-μL reaction mixture contained 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]GTP, 1.5 mg/mL microtubule protein, and the indicated preparation of pNHppA at 1.0 mM. After 10 min at 37 °C, an aliquot of each sample was processed as described in the text. The amounts of ATP and P_i formed were quantitated and expressed as nanomoles formed per milliliter of reaction.

commercial preparations, and the repurified pNHppA, are compared to the amount of ATP formed when each preparation (at a nominal concentration of 1.0 mM—i.e., not corrected for impurities) was mixed with microtubule protein and [γ-^{32}P]GTP. There was good correlation between the amount of ATP formed and the amount of ADP contamination present in the commercial preparations;[6] and quantitatively, the amounts of ATP formed are in accord with the amounts of contaminating ADP. For example, with preparation 5, "1.0 mM" pNHppA would result in 39 μM ADP in the reaction mixture, accounting for the 25 nmol/mL ATP formed. In addition, the amount of P_i formed was inversely related to the purity of the pNHppA preparations. It should also be noted that the variable contamination observed in commercial pNHppA preparations is not likely to result from storage. Both the chromatographic analysis of the repurified pNHppA (Figure 5) and the study with [γ-^{32}P]GTP (Table III) were performed after 8 months of storage of the analogue at −20 °C.

Does It Matter? In addition to the potential effects of impurities in commercial preparations of pNHppA on the precise quantitation of GTP hydrolysis by microtubule protein, we were concerned that pNHppA might alter the basic assembly/hydrolysis reaction, in view of growing evidence that adenine nucleotides can interact with tubulin (Penningroth & Kirschner, 1978; Jameson & Caplow, 1980; Zabrecky & Cole, 1980; Duanmu et al., 1986). Carlier and Pantaloni (1982) had indicated that the addition of 1.0 mM pNHppA + 1.0 mM $MgCl_2$ to microtubule protein + GTP had minimal effects on microtubule assembly and its associated GTP hydrolysis. We wished to verify this conclusion, especially since we had previously observed reduced GTP hydrolysis at Mg^{2+} concentrations over 1.0 mM (Huang et al., 1985). To eliminate all interference from the nonspecific phosphatases contaminating microtubule protein preparations, we examined the effects of these two components, both singly and in combination, in a system containing purified tubulin and heat-treated MAPs [both proteins are required for GTP hydrolysis in 0.1 M Mes—see Hamel and Lin (1984)]. The experiment presented in Figure 6A demonstrates that either an additional 1.0 mM $MgCl_2$ (supplementing the basic 0.5 mM concentration) or 1.0 mM pNHppA (the repurified preparation was used) inhibited GTP hydrolysis by heat-treated MAPs + tubulin. Greatest inhibition of all (about 75–80%) was observed when both extra $MgCl_2$ and repurified pNHppA were added to the reaction.

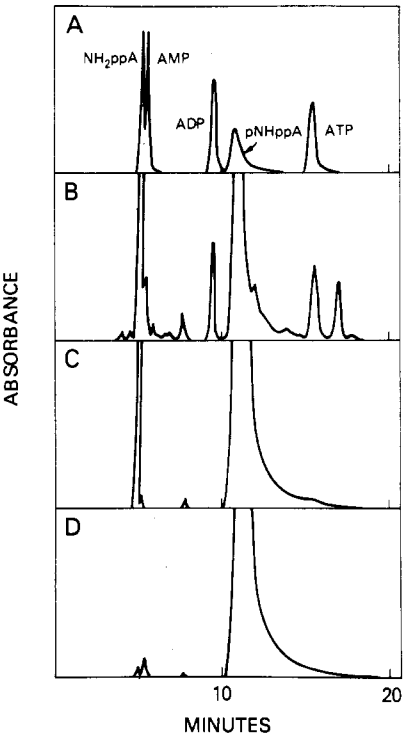

FIGURE 5: Analysis of pNHppA preparations by high-performance liquid chromatography. Chromatography was performed as described in detail in the text. For the analysis presented in panel A, a mixture containing equimolar concentrations (0.1 μmol each) of AMP, ADP, ATP, repurified NH_2ppA, and repurified pNHppA was applied to the column. For the analyses presented in panels B–D, 0.3 μmol of three different pNHppA preparations was applied to the column. In panel B, one of the least pure commercial preparations of pNHppA was used (preparation 5; see Table III), while in panel C, one of the purest commercial preparations of pNHppA was used (preparation 7; see Table III). In panel D, repurified pNHppA (see text) was used. The sensitivity setting of the recorder was 8 times as great in the analyses presented in panels B–D as that presented in panel A.

When microtubule assembly was followed under comparable conditions (Figure 6B), comparing the control system to that supplemented with 1.0 mM each of $MgCl_2$ and repurified pNHppA, the latter reaction was clearly significantly slower and reached a lower turbidity plateau. Moreover, when the reaction temperature was reduced from 37 to 30 °C (Figure 6C), a clear prolongation of the lag phase prior to the onset of polymerization was also observed.

[6] Preparation 1 of pNHppA was used in all experiments described above, except Figure 1. The preparation of the analogue used in Figure 1 was exhausted before the high-performance liquid chromatography analyses could be performed, but it, too, was obtained from supplier A.

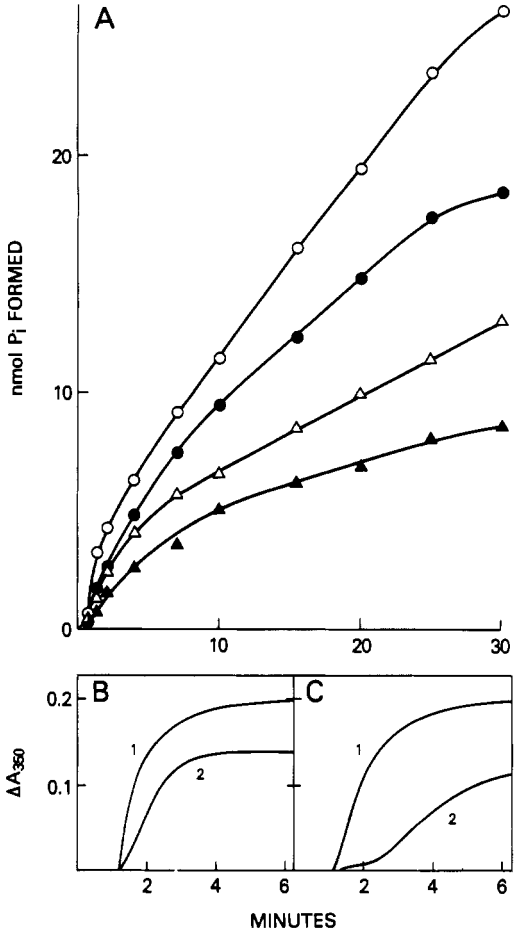

FIGURE 6: Effects of repurified pNHppA and $MgCl_2$, separately and in combination, on GTP hydrolysis and microtubule assembly with tubulin + heat-treated MAPs. (A) GTP hydrolysis. Each 100-μL reaction mixture contained 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]GTP, 1.5 mg/mL purified tubulin, 0.5 mg/mL heat-treated MAPs, and either no further addition (○), an additional 1.0 mM $MgCl_2$ (●), 1.0 mM repurified pNHppA (△), or both an additional 1.0 mM $MgCl_2$ and 1.0 mM repurified pNHppA (▲). At the indicated times at 37 °C, 5-μL aliquots were removed from the reaction mixtures and processed as described in the text. No product except P_i was formed, and its formation was quantitated in nanomoles formed per milliliter of reaction mixture. (B) Microtubule assembly at 37 °C. Each 0.25-mL reaction mixture contained 0.1 M Mes (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM GTP, 1.5 mg/mL purified tubulin, 0.5 mg/mL heat-treated MAPs, and either no further addition (curve 1) or 1.0 mM repurified pNHppA and an additional 1.0 mM $MgCl_2$ (curve 2). The electronic temperature controller was set at 37 °C at zero time. (C) Microtubule assembly at 30 °C. Reaction conditions were identical with those described for panel B, except that the electronic temperature controller was set at 30 °C at zero time.

Conclusion. If pNHppA is to be used as a tool to evaluate GTP hydrolysis in microtubule assembly, great care must be taken to use a preparation with minimal contaminating nucleotides. It is also mandatory to evaluate the reaction for potential products besides P_i. Finally, the preliminary studies presented here with repurified pNHppA, purified tubulin, and heat-treated MAPs demonstrate that it is equally important to evaluate carefully for direct effects of the nucleotide analogue on the precise system being studied.

Registry No. pNHppA, 25612-73-1; GTP, 86-01-1; ATP, 56-65-5; ADP, 58-64-0; NH_2ppA, 47542-69-8; CTP, 65-47-4; UTP, 63-39-8; nucleoside diphosphate kinase, 9026-51-1; AMP kinase, 9013-02-9; GMP kinase, 9026-59-9.

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