A MICROTUBULE ASSOCIATED NUCLEOSIDE DIPHOSPHATE KINASE

by

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ABSTRACT

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Isolated microtubule protein contains a nucleoside diphosphate kinase (NDP kinase) activity which may catalyze a transphosphorylation of tubulin bound GDP to GTP. NDP kinase binds to *in vitro* assembled bovine brain microtubules with the association strongest at early stages of assembly.

NDP kinase was isolated from bovine brain microtubule protein preparations in nanogram amounts. The enzyme is a hexamer of two types of subunits of about 18,000 daltons. The enzyme was isolated, in larger quantities, from bovine brain cytosol without a preliminary

microtubule protein isolation. Both enzymes had the same molecular weights, subunit molecular weights, isoelectric points, catalytic properties, and thermal denaturation profiles and are, therefore, identical or very similiar enzymes. Both subunits of NDP kinase could be reversibly phosphorylated by ATP. Phosphorylation of the enzyme created multiple, more acidic forms that retained activity.

A rabbit anti-NDP kinase antibody was raised to a bovine brain membrane associated NDP kinase. This antibody cross-reacted with the microtubule protein NDP kinase and with the NDP kinase of mouse 3T3 fibroblasts. The antibody did not cross-react with tubulin or with the major microtubule associated proteins. The antibody stained fibrous structures resembling microtubules in 3T3 cells. It is likely, therefore, that NDP kinase is associated with microtubules in cells.

The guanine nucleotide binding site of tubulin was affinity labeled with periodate oxidized GDP. This covalently attached nucleotide could be phosphorylated showing that a true transphosphorylation, which might be catalyzed by NDP kinase, mechanism does exist. This experimental design should permit good mechanistic studies of transphosphorylation and of the role nucleotides play in microtubule assembly.

For My Father

James Warren Nickerson

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INTRODUCTION

The traditional concept of the cell in biochemistry is a liquid cytoplasmic soup in which membrane-delineated organelles float. The chemistry of the cell, enzymatic and otherwise, is assumed to be solution chemistry with substrates, products, enzymes, and regulators all bouncing around randomly, obeying the simple rules of solution kinetics. This view still is a necessary simplification because it allows us to write simple equations modeling the behavior of enzymes, but we must remember that it is only an assumed simplification.

Recent advances in our understanding of the cell show it to be a much more ordered and structured entity than we had ever imagined. It seems more and more likely that even metabolism has a structure, with the enzymes of metabolic pathways held in specific positions and orientations in a cytoplasm that is a dense array of fibers and fiber associated structures. Perhaps solid state chemistry is a more appropriate paradigm for cellular chemistry than is solution chemistry. It is because of this change in our thinking that I believe that the biochemistry of cytoplasmic structure will come

to dominate our thinking and research about cellular metabolism in the not-too-distant future.

For my thesis research, I have chosen to study one of the best characterized structural systems in the cytoplasm, the microtubule. The principle microtubule protein, tubulin, tightly binds two guanine nucleotides whose functions are not known. In early studies on microtubules we discovered, or perhaps re-discovered, the presence of nucleoside diphosphate kinase in preparations of microtubule protein. It had previously been proposed that this enzyme might catalyze a reaction phosphorylating a tubulin bound GDP to GTP *in situ* and by this mechanism it might regulate some aspect of microtubule assembly or function. We decided to study this enzyme. We wished to isolate and characterize the NDP kinase from microtubule protein preparations and to develop methods of producing it in sufficient quantities for further biochemical study. We wanted to determine whether this enzyme actually binds to microtubules both *in vitro* and in the cell. Finally, we wanted to know whether this enzyme might catalyze the phosphorylation of tubulin bound GDP. We saw this work as a foundation and hoped that it would pave the way to detailed biochemical studies of the role NDP kinase might play in the assembly or function of microtubules.

CHAPTER 1

LITERATURE REVIEW

MICROTUBULES

Electron microscopists and cell biologists have, over the last few decades, identified a filamentous structure within the cytoplasm of eukaryotic cells consisting of at least four filament types, each distinguished by a characteristic diameter in electron micrographs of cell cross-sections. The smallest of these are the F-actin containing microfilaments with a diameter of 60 A (1). Intermediate filaments all have a diameter of 100 A but biochemical characterization has shown that there are at least 5 different types, each with a unique protein composition (2). Myosin filaments are distinguished by their diameter of 150 A (3). Largest, first discovered, and most important of all for the purposes of this Thesis are the microtubules measuring 250 A. The development of more advanced methods for extracting, fixing, and observing the cytoskeleton have revealed a much more complex structure than had been imagined, containing many more types of filaments and structures than the four that we have been discussing (4,5). Nevertheless, these four filament types remain the only biochemically well characterized cytoplasmic structural elements.

Microtubules are involved in cell motility, intracellular motility, and in the maintenance of cell structure. The properties of microtubules and their role

in these various phenomena have been extensively reviewed (6-8). The main component of microtubules is tubulin, a protein heterodimer of two similiar subunits of 52,000 to 55,000 daltons (9). Microtubules also contain a variety of other proteins, the identity of which may vary from cell type to cell type. If these proteins can be co-isolated with tubulin, then they are refered to as microtubule associated proteins or MAPs. The structure and properties of microtubules vary in different cell types and even between different classes of microtubules in the same cell. Some of these differences in microtubule properties include changes in ultrastructure, for example the unusual and characteristic double-tubule structure of outer doublet microtubules in cilia and flagella (10), and differences in cold stability (11), with some microtubules undergoing depolymerization at 4°C. Since tubulin is the major component of all these microtubules it seems likely that these differences in properties are caused by the MAPs.

Microtubule Reconstitution In Vitro - Microtubule proteins have been isolated from a wide variety of species and tissues, but most biochemical studies have been done on the microtubule proteins of either bovine or porcine brain isolated by cycles of assembly-disassembly. Microtubule proteins can be assembled into microtubules in vitro by incubation at pH 6.3-7.0 and at temperatures greater than

25°C in the presence of GTP and in the absence of Ca^{2+}. These microtubules can then be disassembled by decreasing the temperature to less than 20°C. Taking advantage of these properties, microtubule proteins can be isolated from brain by cycles of assembly-disassembly (12-14). Microtubules are assembled from a brain high speed supernatant by incubation with GTP and the Ca^{2+} chelator EGTA at temperatures above 25°C. Microtubules are harvested by warm centrifugation, resuspended in the cold to disassemble microtubules, and centrifuged in the cold to remove aggregated material. This represents a single cycle of assembly-dissassembly. Two or three cycles are generally used to isolate microtubule protein for biochemical studies.

These isolation procedures depend on the ability to reconstitute microtubules from their constituent proteins. Microtubule assembly is not a simple polymerization, but is instead a two step initiation-elongation process (15-17). In the first step, initiation complexes or nucleation centers are formed from microtubule proteins. In the second step of assembly, elongation, microtubules grow from these nucleation centers. As predicted on theoretical grounds (18), there is a critical concentration of microtubule protein required for assembly. At protein concentrations below this critical value assembly will not occur.

Microtubule assembly <u>in vitro</u> does not reach

equilibrium; it reaches steady state. At one end of the microtubule, polymerization is occuring, while at the opposite end, the microtubule is depolymerizing. At steady state, the rate of assembly at one end is equal to the rate of disassembly at the other. This opposite end assembly-disassembly is called `treadmilling' and was first described by Leslie Wilson and his coworkers (19,20). It is not known whether treadmilling occurs *in vivo*. The fact that microtubules in the cell are anchored in microtubule organizing centers suggests that the disassembling end of the microtubule might not be free to engage in disassembly reactions (21-23).

Microtubule Associated Proteins - Proteins that co-isolate with tubulin through cycles of assembly-disassembly are operationally defined as microtubule associated proteins (MAPs). To prove that they are true microtubule proteins, that is structural components of microtubules, it should be shown that these proteins are associated with microtubules in cells. This can generally be done by immunocytochemical methods (24-26).

Microtubule protein isolated from bovine or porcine brain by cycles of assembly-disassembly consists of 75-90 % tubulin by mass and 10-25 % MAPs (12-14, 27,28). The most prominant of the MAPs are the high molecular weight proteins MAP1 and MAP2 with molecular masses of more than 300,000 daltons. These proteins, both of which

have been isolated and characterized, associate with the microtubule as projections protruding from the surface at regular intervals along its length (29-31). A second group of MAPs are a group of proteins of 70,000 to 80,000 daltons known as tau proteins (29,32,33). One of these, known as the tubulin assembly protein, has been isolated and can also be shown to associate with microtubules in cells (26), although its exact structural location on the microtubule is unknown.

The function of the high molecular MAPs and tau proteins is not well understood. These proteins have been shown to promote the in vitro assembly of tubulin (29,26,34) although they are not necessary for assembly (35,36). It has been suggested that the MAPs may be the binding sites for organelle attachment to microtubules (37). MAP1 and MAP2 are the most likely candidates for filling this role because, as we have discussed, they exist as projections from the microtubule surface. It has also been suggested the MAPs may be the cross-bridges between microtubules and other cytoskeletal structural elements such as actin filaments (38) or intermediate filaments (39). There is actually some evidence for this latter speculation. Both MAP2 isolated without a heating step and several of the tau proteins cross-link F-actin in vitro forming a gel of actin filaments (38). MAP2 isolated with a heating step organizes purified actin filaments into discrete bundles which are composed of

20 % MAP2 and 80 % actin (40). Since MAP2 binds to both microtubules and to F-actin it might easily cross-link the two in the cell. Both MAP1 and MAP 2 bind to neurofilaments, the intermediate filaments of neurons (41), but neither bind to the intermediate filaments of astroglial cells (41). Co-localization in cells of MAP2 and the intermediate filament protein vimentin has been found in primary cultures of rat brain (39). As a result of these studies it seems likely that the most commonly studied MAPs, tau and the high molecular weight MAPs, are structural elements mediating the interaction of the microtubule with other cytoskeletal elements.

Microtubule Associated Enzymes - There is a second group of proteins that have been found in preparations of brain microtubule protein isolated by cycles of assembly-disassembly, the microtubule associated enzymes. Since co-persistance with tubulin through such cycles is circumstantial evidence of *in vivo* association with microtubules these proteins deserve mention in any discussion of microtubule biochemistry, but because the microtubule associated enzymes are generally present at much lower stoichiometric ratios with tubulin they are more difficult to study and, as a result, frequently overlooked. Among these enzymes are diglyceride kinase (42), a cAMP-dependent protein kinase (42-45), phospholipase C (46), an ATPase (42,47,48), adenylate

kinase(49), and nucleoside diphosphate kinase (49-51).

The microtubule associated enzymes have a variety of catalytic activities and they might be microtubule associated for one of at least two reasons. First, their catalytic activities could be involved in microtubule function or in the regulation of microtubule function. Thus, a protein kinase associated with microtubules might phosphorylate a microtubule protein, changing its structure or function. The second possibility is that binding to microtubules is a method for regulating the function of the enzyme. Numerous examples of ambiquitous enzymes , enzymes that are regulated by changing their subcellular localization, are known (52). Binding to cellular organelles may change their catalytic properties or their access to substrates.

The best characterized of the microtubule associated enzymes is the cAMP-dependent protein kinase, whose structure and activity may provide us with clues about what to expect from other microtubule associated enzymes. The protein kinase exists as a complex of three polypeptide chains, a catalytic subunit, a regulatory and cAMP-binding subunit, and MAP2 (53). It is likely that the catalytic and regulatory subunits are located on the arm of MAP2 that projects from the microtubule surface (53,54). The principal microtubule protein substrate for phosphorylation by this protein kinase is MAP2 itself (43-45,53). The phosphorylation of microtubule proteins by

this protein kinase may inhibit the rate of microtubule `treadmilling', that is polymerization at one end of the microtubule and depolymerization at the other at steady-state (19,20). In summary, the microtubule associated cAMP-dependent protein kinase exists as a complex with a structural component of the microtubule-MAP2, which also serves as its principal substrate, and may regulate microtubule assembly or disassembly events.

There is sufficient evidence in the case of the protein kinase to show that it is in fact a microtubule associated protein and that it is probably important in microtubule function. In the case of the other enzymatic activities found in microtubule protein preparations, I believe there is as yet insufficient evidence to classify them as truely microtubule associated. To better focus our studies on these enzymes we should establish criteria for concluding that a particular enzyme is microtubule associated. More importantly, we should decide whether the <u>in vitro</u> demonstration of microtubule association is either sufficient or necessary for establishing the importance of an enzyme in microtubule function.

The criterion that is most commonly used is that of co-purification of the protein with tubulin through many cycles of assembly-disassembly. Proteins which bind to microtubules tightly, that is with very low dissociation constants, will, after several cycles, pass through subsequent cycles at an <u>apparent</u> constant stoichiometry

with tubulin. But, let us imagine the existance of a microtubule protein that binds to microtubules, although less tightly. In this case the stoichiometric ratio of the protein to tubulin may decrease from assembly cycle to cycle. If the binding constant is sufficiently low the amount of the protein that is present may become unmeasureably low after several cycles. I see no reason to assume that any protein that is important in microtubule function must bind tightly enough to co-purify through cycles of assembly-disassembly, especially at the sub-physiological protein concentrations used in cycling. This operational definition of microtubule associatedness was useful in the early days of microtubule research since it helped to identify the most likely and easily studied MAPs, but it does not represent a logical imperative.

What guidelines should we use to prove that an enzyme is microtubule associated? I believe that there are three criteria to be satisfied. A microtubule associated enzyme should bind to microtubules <u>in vitro</u> even if the binding is low affinity or at a low stoichiometry to tubulin. Second, it should be demonstrated that the enzyme associates with microtubules in cells. Third, there should be some functional significance to the association with microtubules. Either, binding to microtubules should affect the catalytic properties of the enzyme, or it should have some role in microtubule function.

Rings and Things - Preparations of microtubule protein which are made by cycles of assembly-dissassembly contain tubulin in two forms, as 6s dimers and as ring-shaped oligomers of 30-36s (this is reviewed in 55). Oligomeric microtubule protein rings also contain MAPs, especially the high molecular weight proteins MAP1 and MAP2, and differ in structure according to whether the microtubule protein was prepared in the presence or in the absence of glycerol (27,55). Microtubule protein prepared in the presence of glycerol contains rings that are 36s double-walled structures containing 42 tubulin subunits (55,56), while microtubule protein prepared in the absence of glycerol contains 18s rings with 29 tubulin subunits and 30s rings that consist of two stacked 18s rings (55,57). These 18s and 30s rings are interconvertable and exist in equilibrium. Typically, 25-75% of the protein in a microtubule protein preparation isolated by two cycles of assembly exists in oligomeric rings, depending on solution conditions and protein concentration. While MAPs are present in rings they are not necessary. Pure tubulin can form rings that resemble the 36s rings discussed above (58), although, of course, they contain no MAPs.

It has been demonstrated that the tubulin of rings and the tubulin that exists as free dimers are not quickly interconverted, nor are they in a simple dimer ring equilibrium (reviewed in 55). Weingarten et al. (32)

showed that in microtubule protein preparations isolated in the presence of glycerol, 6s and 36s forms of tubulin did not interconvert even during a complete cycle of assembly-disassembly. Thus, tubulin that was in the 6s form before assembly was again in the 6s form after disassembly and tubulin that started as 36s was again 36s after disassembly. The 6s and 36s forms could be interconverted only at high ionic strength, although 6s tubulin could be converted to 36s by the addition of excess tau protein. Other studies by Zeeberg *et al.* (59) and by Pantaloni *et al.* (60) show that there is a very slow exchange of subunits between 36s rings and 6s dimers. 36s rings are direct, cold induced disassembly products of microtubules; they are not formed from microtubule released dimers (59), but once rings and dimers are produced by cold disassembly they exchange subunits with a half time of about 90 minutes (60).

Studies of *in vitro* microtubule assembly by synchrotron radiation scattering have shown that oligomeric rings are not nucleation centers for assembly; rather, they are disassembled into protofilament fragments which nucleate assembly (61-63). The tubulin subunits which appear in microtubules during the early stages of assembly come preferentially from 36s rings (60). This has been shown with assembly experiments starting with [^{125}I]-tubulin in dimers and unlabeled tubulin in rings (60). It seems unlikely that rings are directly involved

in microtubule elongation events (61-63) although ring fragments could again be involved. It is not known whether oligomeric rings exist in vivo or, if they do, what their role might be in the cell. Since rings are cold induced depolymerization products that are broken into smaller fragments upon warming (60-63,78), I speculate that ring fragments may exist in vivo where they may be involved in microubule assembly reactions.

MICROTUBULES AND NUCLEOTIDES

Tubulin prepared from brain by either the chromatographic procedures of Weisenberg et al. (64) or by cycles of assembly-disassembly (12-14) contains a mixture of bound guanine nucleotides, mostly GTP and GDP. There are two distinguishable nucleotide binding sites on each tubulin dimer (64-67). At the `non-exchangeable' site (N-site) the nucleotide is very tightly, though non-covalently, bound and does not exchange with guanine nucleotides in solution. At the `exchangeable' site (E-site) the nucleotide is less tightly bound and may exchange with GTP or GDP in solution, although, as we shall see, only under some conditions.

Early estimates of the stoichiometry of nucleotide binding to isolated tubulin (66-68) were 1 mole of guanine nucleotide per mole of tubulin dimer, but these were unreliable estimates because the methods used to extract nucleotides did so incompletely, removing nucleotide only from the E-site (69,70). Subsequent work by Weisenberg et al. (69,70) showed that isolated microtubules contain 2 moles of guanine nucleotide per mole of tubulin dimer. All of the E-site nucleotide is unremovable under non-denaturing conditions and is GDP after microtubule disassembly (68,69,71). Most of the N-site nucleotide on the tubulin dimer is GTP (49,68), while on the assembling microtubule the E-site nucleotide starts as GTP, but is

hydrolyzed to GDP as assembly proceeds (71-73).

The binding of GTP to the E-site of the dimer requires a free, reduced sulfhydryl group at the binding site (74) and requires a metal ion: Mg^{2+}, Ca^{2+}, or Cr^{3+} (74,75). This suggests that the species binding to the microtubule is a GTP-metal ion complex, for example Mg^{2+}-GTP. The dissociation constant for GTP binding to the E-site on the tubulin dimer has been measured and most estimates cluster around 0.5×10^{-6} M (74,76,77). The dissociation constant for GDP binding to the dimer E-site may be as high as 2.0×10^{-6} M (74) and as low as 0.15×10^{-6} M (77). The guanine nucleotide bound to the E-site on microtubule protein oligomeric rings is, however, much more tightly bound and does not detectably exchange with nucleotides in solution (59,69). In fact, Zeeberg et al. (59) have shown that the rate of nucleotide exchange on 36s rings is the same as the rate of tubulin subunit exchange and, as we have seen, this occurs with a half time of about 90 minutes (60). The molecular basis for this difference in exchangeability between oligomeric and dimer binding sites is not known.

Many studies have suggested that the E-site is specific for the binding of guanine nucleotides and their analogues (49,66,74). In a more thorough investigation, Penningroth and Kirshner (79) have shown that, when tightly bound GDP is removed, the E-site has a broad specificity for both ribo- and deoxyribo- nucleotides.

The much higher affinity of guanine nucleotide binding, however, makes it likely that they are the only nucleotides bound *in vivo*.

The *in vitro* assembly of microtubule protein in nucleotide free buffers has only been achieved with a high concentration of glycerol or sucrose (12,80), when the E-site has been preloaded with GTP (80), or when a GTP regenerating system has been included in the polymerization mixture (80-83). It seems clear that a nucleoside triphosphate must occupy the tubulin E-site at some point in the assembly process in order to support in vitro microtubule assembly.

The most fundamental question about nucleotides and microtubules is: why is a nucleotide required at all? It has been reported that tubulin dimers with E-site GDP can participate in microtubule elongation but not in assembly initiation events (84), although the rate of elongation in the presence of GDP is 100 fold slower than with GTP (84). Penningroth and Kirschner (79) have reported that assembly proceeds with sub-stoichiometric amounts of E-site nucleotide, suggesting that tubulin can participate in elongation even in the absence of any E-site nucleotide.

Hydrolysis of E-site GTP with the release of inorganic phosphate accompanies assembly (49,68,69,71,72,83,85), but studies with non-hydrolyzable analogues of GTP show that the energy of hydrolysis is not necessary for assembly.

Both guanosine 5'-[β,γ-methylenetriphosphate] or GMP-PCP (49,86) and guanosine 5'-[β,γ-imido- triphosphate] or GMP-PNP (69,70,86-88) have been used to induce assembly. It has frequently been reported that microtubules formed with these non-hydrolyzable GTP analogues are resistant to Ca^{2+} induced disassembly (69,87,89), but in these studies microtubule assembly and disassembly have been measured by by changes in turbidity. Penningroth and Kirschner (49) studied this more carefully and found that although the turbidity did not decrease much after treating analogue assembled microtubules with Ca^{2+}, no Ca^{2+} stable microtubules could be found by electron microscopy. It may be that turbidity measurements have given an artifactually high estimate of microtubule stability in these studies. Microtubules assembled with GMP-PCP (49) or with the non-hydrolyzable analogue Cr^{3+}GTP (75,90) are more cold stable than those assembled with GTP and this has been verified by electron microscopy (49). Microtubules assembled with non-hydrolyzable analogues are also more resistant to dilution- (91) and podophyllotoxin- (86) induced disassembly. These studies show that the energy of GTP hydrolysis is not necessary for assembly. Despite some methodological problems, I think it can be concluded that GTP hydrolysis somewhat destabilizes the microtubule, perhaps by increasing the rate of reactions at the disassembling end.

The best idea so far presented for why GTP and GTP hydrolysis are used in the mechanism of assembly has been advanced by Kirschner (92,see also 93,94). He has proposed that GTP hydrolysis during the assembly process is part of the mechanism by which the cell selectively stabilizes properly anchored microtubules and prevents the formation of spontaneously assembled microtubules, thus providing the cell with a way to control its cytoskeletal geometry. The evidence in support of this hypothesis is only theoretical. An unanchored microtubule at steady state is `treadmilling', that is assembling at one end and disassembling at the other. At the assembling end tubulin molecules contain GTP, while at the disassembling end they contain GDP. Thus the equilibrium constants for tubulin addition to the microtubule can be different at each end without violating the statistical mechanical principle of microscopic reversibility. The result of this, as shown by Kirschner (92), is that the critical concentration of tubulin required for assembly is different at both ends of the microtubule; so, there exists a range of tubulin concentrations at which disassembly at one end will be faster than assembly at the other end. By maintaining a free tubulin concentration in this range, the cell can prevent the spontaneous formation of unanchored microtubules, leaving only those microtubules whose disassembling end is anchored in an appropriate organizing center.

There are two mechanisms by which E-site GTP might be generated to support microtubule assembly: nucleotide exchange and the transphosphorylation of E-site GDP to GTP without exchange.

Exchange:

$$\text{Tubulin-GDP} + {}^{*}\text{GTP} \rightleftharpoons \text{Tubulin-}{}^{*}\text{GTP} + \text{GDP}$$

Transphosphorylation:

$$\text{Tubulin-GDP} + {}^{*}\text{NTP} \rightleftharpoons \text{Tubulin-GTP} + {}^{*}\text{NDP}$$

A transphosphorylation mechanism could be driven by any nucleoside triphosphate (NTP) as pictured above.

Under conditions of pH, ionic strength, and metal ion concentration favoring assembly, ATP and every other nucleoside triphosphate tried will induce microtubule assembly (12,49,69,77), even though binding of these nucleotides to tubulin cannot be detected. Michael Jacobs and his coworkers (77,95) were the first to suggest that a transphorylase activity in microtubule protein preparations transfers the gamma phosphate of NTP to a tightly bound tubulin-GDP, leaving a GTP bound to the tubulin E-site. This tubulin-GTP would then be capable of participating in assembly reactions requiring GTP.

It has been suggested that the proposed

transphosphorylation reaction might be catalyzed by a nucleoside diphosphate kinase (49,50) and the two terms have sometimes been used interchangeably. I think that it is important to make a distinction between the two terms. Thus nucleoside diphosphate kinase (NDP kinase) catalyzes the phosphorylation of a free NDP in solution to NTP while transphorylase uses tubulin-GDP as a substrate, phosphorylating GDP in situ to GTP. An NDP kinase could support ATP induced assembly by phosphorylating GDP in solution to GTP which would exchange with a GDP bound to the tubulin dimer.

Mechanism 1

NDP Kinase: $ATP + GDP \rightleftharpoons ADP + GTP$

Exchange: $GTP + Tubulin\text{-}{}^{*}GDP \rightleftharpoons {}^{*}GDP + Tubulin\text{-}GTP$

A transphosphorylase could support ATP induced assembly by phosphorylating tubulin bound GDP in situ.

Mechanism 2

Transphosphorylation:

$$ATP + Tubulin\text{-}GDP \rightleftharpoons ADP + Tubulin\text{-}GTP$$

It is possible that transphosphorylation, if it actually occurs, and nucleoside diphosphate kinase could be two activities of the same enzyme but there is no reason to assume that they are *a priori*.

Penningroth and Kirshner (49) demonstrated that microtubule protein preparations contain a true transphorylase activity which can phosphorylate tubulin E-site bound GDP *in situ*. They incubated tubulin containing bound GDP with a mixture of [^{3}H] GTP and [gamma-^{32}P] UTP under conditions that would support assembly. They found that the ^{32}P labeled nucleotide appeared on the tubulin dimer almost 10 times faster than the ^{3}H labeled nucleotide. Thus, they concluded that transphosphorylation is occuring 10 times faster than simple nucleotide exchange. The validity of this experiment has been challenged by Brylawski and Caplow (96) who have suggested that it is impossible to remove free nucleotide from microtubule protein preparations and so the results of the double label experiment are complicated by the presence of unlabeled guanine nucleotides diluting the specific radioactivity of the [^{3}H] GTP pool. Jacobs and Huitorel (50) have performed a similiar double label experiment with [^{3}H] GDP and [gamma-^{32}P] GTP. Tubulin was preloaded with [^{3}H] GDP and then incubated with [gamma-^{32}P] GTP. The nucleotide

bound to the E-sites on dimers was almost all ^{32}P labeled while the E-sites on 36s rings was both ^{3}H and ^{32}P labeled. This experiment suggests that E-site GTP on dimers is produced by exchange while E-site GTP on rings is produced by transphosphorylation. This result cannot be dismissed as an artifact of isotope dilution, but, does not finally settle the debate. NDP kinase is associated with the 36s rings and for reasons of simple propinquity may preferentially phosphorylate GDP being released from nearby ring E-sites.

Penningroth and Kirshner (49) presented evidence that the transphosphorylase activity is not intrinsic to the tubulin dimer. The transphosphorylase activity can be separated from the commonly studied MAPs, the high molecular weight MAPs and tau proteins, by chromatographic procedures (49). It seems likely that microtubule protein preparations contain a previously unstudied MAP or microtubule associated enzyme which is the transphorylase.

Some early work suggested that the N-site GDP was phosphorylated by the transphorylase (66) but the method used to distinguish E-site and N-site nucleotide did not work. It has now been convincingly demonstrated that phosphorylated nucleotide ends up on the E-site (49,50). Thus, the transphosphorylase, if it exists, is phosphorylating E-site GDP.

The issue of whether a transphosphorylase exists is further complicated by the fact that simple exchange of

E-site nucleotide can support microtubule assembly. For example, the non-hydrolyzable nucleotide analogues GMP-PCP and GMP-PNP (49,69,70,86-88) and the non-hydrolyzable Cr^{3+}GTP complex (75,90) can both support *in vitro* microtubule assembly. Further, the rate of GDP release from the E-site during assembly may be fast enough to support ATP catalyzed assembly by an NDP kinase mechanism, without transphosphorylation (97). If the Penningroth and Kirshner (49) experiments discussed above are valid then transphosphorylase generated GTP can appear at the E-site an order of magnitude more rapidly than GTP in solution can exchange onto the E-site. It is possible that, while simple exchange *can* support microtubule assembly, transphosphorylation is the normal method for generating E-site GTP for assembly. I suggest that the transphosphorylation may exist for the generation of E-site GTP on rings and other oligomers that may be polymerization intermediates such as protofilament fragments (61-63,84), where the nucleotide is not readily exchangeable (50,69). Whether this mechanism has any relationship to microtubule function in the cell is unknown and, given our current state of ignorance, practically beyond speculation. We can only fall back on a basic tenet of the faith and believe that any process or structure that we can demonstrate *in vitro* must have some relevance *in vivo*.

This discussion shows that NDP kinase might be the

putative transphosphorylase in microtubule protein preparations and might play an important role in microtubule assembly. This discussion highlights the important questions to be answered about microtubule preparation NDP kinase. Is NDP kinase truely microtubule associated? Does a transphosphorylation reaction actually occur? Is that reaction catalyzed by NDP kinase? If we could answer these questions we might know something important about the role of nucleotides in microtubule assembly.

NUCLEOSIDE DIPHOSPHATE KINASE

Nucleoside diphosphate kinase (NDP kinase) has been studied from a wide variety of sources: plant, animal, and prokaryotic (for a review see 98), where they are usually cytosolic (99-105), mitochondrial(105-109), or associated with the plasma membrane (110,111). These enzymes transfer a phosphate from a nucleoside triphosphate to a nucleoside diphosphate and are not very substrate specific for either nucleotide. NDP kinase from most sources has subunits of 16,000 to 20,000 daltons and exists either as a tetramer of about 80,000 daltons or as hexamers of about 100,000 daltons. The only NDP kinase that doesn't quite fit into this size range is the 2s enzyme of Chlamydomonas flagella (112), which, judging by its small sedimentation coefficient, is considerably smaller.

Those NDP kinases whose catalytic properties have been studied catalyze double displacement (ping-pong) reactions (98,99,102,108,113). Based on these kinetic results we would predict that NDP kinases should have phosphorylated intermediates, high energy phosphate-enzyme complexes. In fact, such phosphorylated forms of NDP kinase have been detected (98,101,114-117) and the phosphorylated amino acid is a histidine (98,101,114,115).

The nucleotide specificity of NDP kinases is not very high and varies from enzyme to enzyme (98). All NDP kinases have a requirement for a divalent metal ion,

especially Mg^{2+} and Mn^{+}, so it seems likely that the actual substrates for NDP kinases are Mg^{2+}NTP and Mg^{2+}NDP (98,100,102).

NDP kinases from most sources come in multiple isozymes (100,102,105,118). Since, the enzymes are not known to contain carbohydrate it has been proposed that the different isozymes represent different polypeptide sequences (118). Studies showing multiple isozymes have frequently been done by isoelectric focusing of tissue homogenates or crude extracts (105,118). It has not previously been suggested that some of the isozymic forms of NDP kinase could be phosphorylated intermediates,so let me suggest it here. A single hexameric NDP kinase molecule contains 6 active sites and so could be phosphorylated at 6 positions. The addition of 6 phosphates to the molecule could profoundly lower the molecules pI. Many phosphorylation permutations are possible, containing 0 to 6 phosphates, and could represent many isozymes.

A bovine brain NDP kinase has been isolated and studied by Robinson et al. (119). This enzyme is isolated from the particulate fraction of brain, is apparently membrane associated, and is solubilized in the isolation procedure by the detergent BRIJ 56. The isolation procedure is novel, relying on an unusual property of this NDP kinase, its ability to compete with BRIJ 56 micelles for binding to the ligand of Blue Sepharose. This NDP

kinase is a hexamer of apparently identical 16,600 dalton subunits, functions with a phosphorylated intermediate, and has a pI of 8.4. The membrane localization of this NDP kinase suggests that it may not be the NDP kinase which is present in microtubule protein preparations.

As discussed earlier in this chapter, NDP kinase activity has been found in preparations of microtubule protein prepared by cycles of assembly-disassembly (49,50,97). It has been shown that this NDP kinase is not of mitochondrial origin and not released from organelles by homogenization (120).

Nucleoside diphosphate kinase activity has been found in proximity to microtubules in cilia and flagella. NDP kinase activity is associated with the outer fiber microtubules of sea urchin sperm flagella (121) and Tetrahymena cilia (122). NDP kinase associated with Chlamydomonas flagella microtubules can be seperated into a 2s enzyme that is exclusively flagellar and several 4.3s isozymes that are present both in the flagella and in the cell body (112).

The most endearing of NDP kinase's characteristics is its memory. Studies of the bovine liver mitochondrial NDP kinase (123) show that the conformations of the enzyme phosphorylated intermediate vary according to the identity of the donor nucleotide. Thus, the affinity of the enzyme intermediate for a particular NDP depends on the identity of the NTP which provided the high energy phosphate.

These affinities for NDPs may vary by as much as 10-fold. This is important to keep in mind when studying the kinetic properties of NDP kinases; kinetic constants may be meaningless alone and must be seen as partially dependent upon the catalytic history of the enzyme.

GTP AS A REGULATORY MOLECULE

I have suggested that NDP kinase may regulate microtubule assembly or function- a regulation mediated by guanine nucleotides. While GTP can serve as a substitute for ATP in some reactions, providing energy or high energy phosphates, it can also function as a regulator of metabolic processes. Studies in recent years of such systems suggest a principle of nucleotide specificity; when the cell wants a nucleotide for energy it uses ATP, but when it wants a nucleotide for regulation it uses GTP. While there are certainly exceptions to this principle, it is valid often enough to be useful.

Probably the best studied use of GTP in a regulatory system is with the G proteins in plasma membrane. These G proteins are guanine nucleotide binding membrane proteins that mediate hormone dependent inhibition or stimulation of adenylate cyclase (124-128) and a different GTP binding protein, transducin, regulates the light-dependent cGMP-specific phosphodiesterase in retina (124,125,129,130). These GTP binding proteins exist as multisubunit complexes when they bind GDP. They remain inactive until they bind GTP and dissociate into subunits which are active regulators of adenylate cyclase and phospodiesterase.

The regulation of chain elongation during protein synthesis involves a factor, Tu, which binds GTP and is

activated by that binding (131). GTP binding proteins may regulate Ca^{2+} channels in mast cells (132) and may also mediate the effects of insulin-receptor binding on a plasma membrane protein kinase (133,134).

GTP strongly stimulates glycosylation of microsomal membrane proteins (135-137), even though it is not a substrate in any of the reactions. Incubation with GTP, but not other nucleotides, causes isolated microsomes to fuse and form larger vesicles (138) and increases the permeability of the microsomal membrane to proteins and sugar nucleotides (139). It has been suggested that the increases in microsomal membrane protein glycosylation are caused by this increased permeability of the membranes to sugar phosphates (139).

The protein products of the ras oncogene family are GTP binding proteins of 21,000 daltons named p21 (140). There are oncogenic forms of p21 and non-oncogenic forms found in normal cells. The normal cellular forms of p21 hydrolyze GTP to GDP, but the oncogenic forms have lost most of their GTPase activity (141) suggesting that GTP hydrolysis may be the biochemical event that attenuates the growth promoting effects of p21 in the normal cell.

These examples show that microtubule assembly is not the only process that might be regulated by GTP and GTP hydrolysis. Since GTP in the cell is most easily made by NDP kinase and since transphosphorylation might occur, not just with tubulin-GDP, but with any guanine nucleotide binding protein, NDP kinase and protein-GDP

transphosphorylases might play a role in the regulation of cellular processes. There is circumstantial evidence that NDP kinase may help to regulate plasma membrane adenylate cyclase by providing GTP for the G proteins. There is a clear correlation between the amount of NDP kinase associated with the plasma membrane and the membrane's adenylate cyclase activity (111). Since this study was performed by a former graduate student from this department it must obviously be very well done.

I think that I have shown that there is enough rational to study further the role that NDP kinases might play in the GTP mediated regulation of cellular processes. The presence of NDP kinase in microtubule protein preparations makes microtubules a good system in which to begin that study.

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CHAPTER II

PRELIMINARY STUDIES

INTRODUCTION

Microtubule protein prepared by the assembly-disassembly method of Shelanski *et al*. (1) contains several enzymes that may be microtubule associated and that might affect microtubule structure or function. The best studied of these microtubule associated enzymes is a cAMP dependent protein kinase which is capable of phosphorylating microtubule proteins (2-4).

The tubulin dimer has two distinguishable guanine nucleotide binding sites, one readily exchanging it's nucleotides with guanine nucleotides in solution (E-site) and the other (N-site) binding it's nucleotides to tightly for exchange to readily occur (reviewed in 5). A transphosphorylation mechanism has been proposed whereby E-site GDP might be phosphorylated to GTP *in situ* (6). It is by this mechanism that ATP and other NTPs might support microtubule assembly without actually binding to the tubulin E-site. It is possible that such a reaction might be catalyzed by a nucleoside diphosphate kinase (NDP kinase) making this an important enzyme to study.

We were interested in studying a microtubule associated enzyme and it's role in microtubule function. The studies presented in this chapter were performed in order to determine whether NDP kinase is microtubule associated and whether it might be worth the effort of further study.

EXPERIMENTAL PROCEDURES

The sodium salt of dTDP, pyruvate kinase, and lactate dehydrogenase were purchased from Boehringer-Mannheim. All other biochemicals were purchased from the Sigma Chemical Company.

Tubulin Preparation - Tubulin was prepared by the method of Shelanski et al. (1) as modified in this laboratory. Bovine brain was homogenized with a Waring Blender in 0.5 ml per gram of brain in Assembly Buffer (20 mM MES, pH 6.4, 70 mM NaCl, 1 mM EGTA, and 0.5 mM $MgCl_2$). The homogenate was centrifuged for 45 minutes at 25,000 x g and then for 45 minutes at 78,000 x g at 4°C. The supernatant was mixed with an equal volume of Assembly Buffer containing 8 M glycerol, 0.5 mM ATP, and 0.5 mM GTP and incubated at 37°C for 45 minutes before centrifuging at 78,000 x g for 45 minutes at 30°C. The pellets were resuspended in AB and were 1 cycle microtubule protein. All further cycles were done similiarly except that the centrifugation steps were done at 105,000 x g for 1 hour. Microtubule protein prepared by 2 cycles of assembly-disassembly is about 90 % tubulin and 10 % microtubule associated proteins as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining (7). Protein

concentrations were measured by the method of Lowry et al. (8).

Nucleoside Diphosphate Kinase Assays - NDP kinase was assayed by a coupled enzyme system similiar to that used by Bergmeyer (9). The assay mixture contained 83.3 mM triethanolamine, pH 7.5, 16.7 mM $MgCl_2$, 67 mM KCl, 1.1 mM phospoenolpyruvate, 5 mM ATP, 0.2 mM NADH, 0.2 units/ml pyruvate kinase, 9 units/ml lactic dehydrogenase, and 0.7 mM dTDP (or 0.32 mM dGDP).

Isotope assays were done by the method of Nakai and Glinsman (10). Microtubule protein was incubated with [gamma-^{32}P] ATP and GDP or dTDP. The NDP kinase reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10 % (w/v). Protein was pelleted at 4^{o}C and the supernatant was extracted 3 times with 3 volumes of diethyl ether and lyophylized. The nucleotides were dissolved in water and spotted onto PEI cellulose plates which were developed in 4 M sodium formate, pH 3.4. Nucleotides were located under ultraviolet light, scraped off of the plates and quantified by liquid scintillation counting.

In Vitro Microtubule Assembly - In vitro assembly of microtubules was performed in Assembly Buffer with 2 cycle microtubule protein at a final concentration of 2-6 mg/ml. Before use, cycle 2 microtubule protein was

centrifuged at 6,500 xg for 5 minutes at 4°C to remove dust and particulate matter in order to reduce background light scattering. Samples were kept at 4°C until they were placed in a Gilford 2400 spectrophotometer with a water jacketed 4 sample chamber maintained at 37°C. Nucleotide additions were made just before placing the samples in the spectrophotometer. Assembly was followed by measuring light scattering at 350 nm (11).

Sucrose Density Gradient Centrifugation - Linear gradients of 30-60 % sucrose in 10 mM MES, pH 6.5, and 1 mM $MgCl_2$ were formed at 30°C. Microtubule protein was assembled in 10 mM MES, pH 6.5, 1 mM GTP, 10 % sucrose, and 1 mM $MgCl_2$ at 30°C. Samples (0.5 ml) were layered on gradients and centrifuged at 38,000 rpm in a Beckman SW41 rotor for 1 hour at 30°C. Immediatly after the run, 0.5 ml fractions were removed sucessively from the top of each gradient with a syringe. Each fraction was assayed for protein concentration and for NDP kinase activity.

RESULTS AND DISCUSSION

The results of *in vitro* polymerization of 2 cycle microtubule protein are shown in Figure 1. Microtubule assembly can apparently be supported better by ATP than it can by GTP. If the nucleotide is added at the same time as the sample is warmed to 37°C, then ATP supported assembly follows GTP supported assembly after a lag of several minutes (Figure 1A). If the nucleotides are preincubated with microtubule protein for 15 minutes at 15°C, a temperature at which there is no assembly, then the lag is eliminated and GTP and ATP supported assembly occur with similiar kinetics. This result suggests that there may be an extra reaction for ATP supported assembly that does not occur with GTP supported assembly and that this reaction may be rate limiting during early stages of microtubule assembly. The most logical candidate for this rate limiting step would be an NDP kinase or transphosphorylase.

We were able to confirm the presence of NDP kinase activity in 2 cycle microtubule protein preparations by both a coupled enzyme assay and an isotopically labeled nucleotide assay as described in "EXPERIMENTAL PROCEDURES". Microtubule protein prepared by two cycles of assembly-disassembly contains about 0.05 units of NDP kinase per mg of protein, that is the amount required to transfer 0.05 umoles of phosphate per minute per mg

Figure 1. Assembly of Microtubules *in vitro* with ATP. The *in vitro* assembly of microtubules was measured by changes in light scattering at 350 nm and 37°C as described in "EXPERIMENTAL PROCEDURES". In the experiment shown in Figure 1A, the nucleotide was added to the assembly mixture just as assembly was begun. In the experiment shown in Figure 1B, the nucleotides were added and incubated with the assembly mixture at 15°C for 15 minutes before the assembly was begun. In both experiments assembly was begun by placing the samples at 37°C and all samples contained 5.6 mg/ml microtubule protein.

Figure 1A

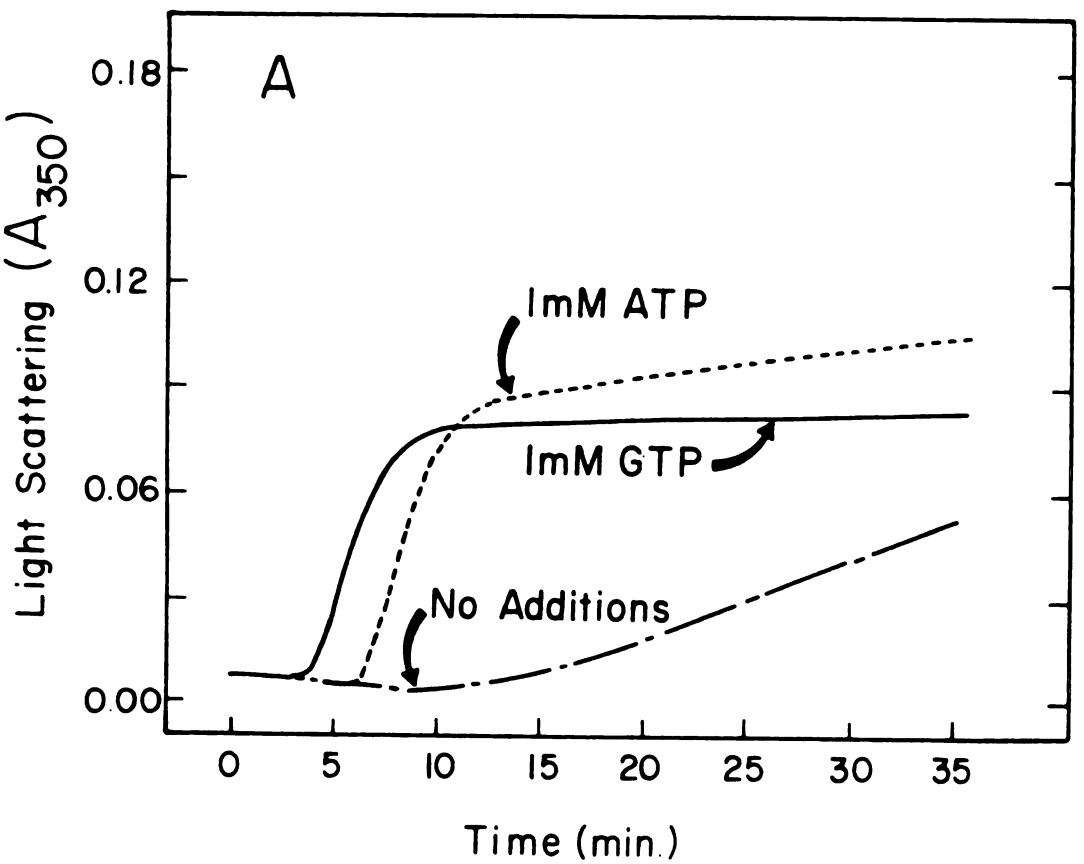
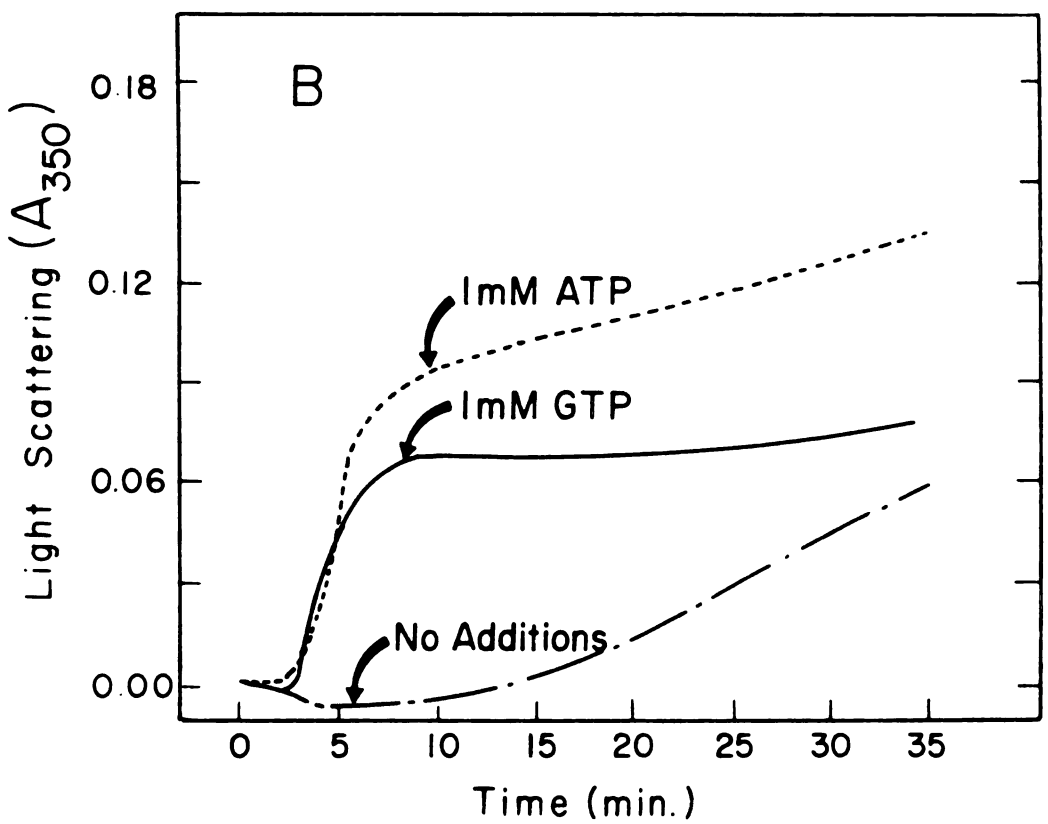

A
0.18
0.12
0.06
0.00
Light Scattering (A_{350})
1mM ATP
1mM GTP
No Additions
0
5
10
15
20
25
30
35
Time (min.)

Figure 1B


B
0.18
0.12
0.06
0.00
Light Scattering (A_{350})
1mM ATP
1mM GTP
No Additions
0
5
10
15
20
25
30
35
Time (min.)

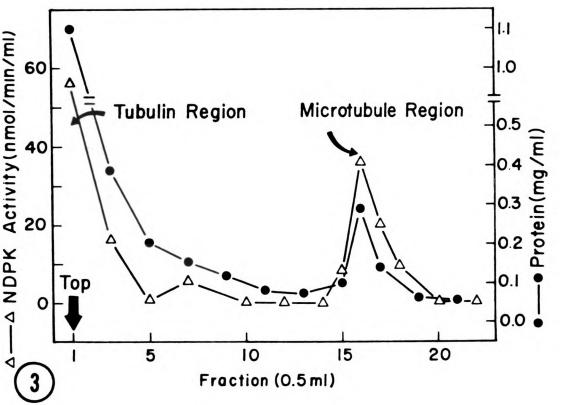
protein at 30oC.

The sucrose density gradient centrifugation of *in vitro* assembled microtubules is shown in Figure 2. There are two major protein bands in sucrose density gradients. The protein band at the top of the gradients should correspond to tubulin dimers and other non-assembled microtubule proteins. The faster sedimenting bands should correspond to intact microtubules, although we have not verified this by electron microscopy. Boehringer-Mannheim bovine liver NDP kinase all sedimented in the top few fractions of an identical gradient (data not shown). In assembled microtubules, most NDP kinase sedimented in the top fractions of the gradient, but the NDP kinase specific activity was highest in the microtubule fractions. This suggests that NDP kinase might be a microtubule protein, an integral constituent of the microtubule.

These early experiments convinced me that NDP kinase might be a microtubule protein, that NDP kinase might be involved in assembly reactions, and that microtubule protein NDP kinase would be an important enzyme to study further in my thesis research.

Figure 2. The association of NDP kinase with sedimenting microtubules. Assembled microtubules were sedimented through linear 30-60 % sucrose gradients in a Beckman SW41 rotor at 38,000 rpm for 1 hour at 30^{o}C. Fractions of 0.5 ml were removed successively from the top of the gradients and assayed for protein concentration and NDP kinase.

Figure 2


Tubulin Region
Microtubule Region
Top
△—△ NDPK Activity(nmol/min/ml)
● Protein(mg/ml)
Fraction (0.5ml)
3

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CHAPTER III

ISOLATION AND CHARACTERIZATION OF THE MICROTUBULE ASSOCIATED NUCLEOSIDE DIPHOSPHATE KINASE

INTRODUCTION

The tubulin dimer has two distinguishable guanine nucleotide binding sites (1). At the "non-exchangeable" (N-site) the nucleotide is tightly, though not covalently, bound and does not exchange with guanine nucleotides in solution (2,3). At the "exchangeable site" (E-site) the nucleotide is less tightly bound and may exchange with free GTP or GDP under certain conditions (2,3). A GTP molecule must be bound at the E-site for *in vitro* microtubule assembly to occur (4) and this GTP is hydrolyzed to GDP during the microtubule elongation phase of assembly (5,6). After microtubule depolymerization there are two mechanisms by which E-site GTP can be regenerated so that the dimer can participate in further assembly reactions: 1) nucleotide exchange with GTP in solution and 2) the transphosphorylation of E-site GDP to GTP without exchange (7-9). Jacobs and Huitorel (9) have shown that the a transphosphorylation catalyzed by some enzyme present in microtubule preparations generates GTP on the dimers of multimeric microtubule protein rings without detectable exchange (9).

Microtubule protein prepared by cycles of assembly-disassembly contains a nucleoside diphosphate kinase (NDP

kinase) (EC 2.7.4.6) activity (7,9-11). This NDP kinase co-assembles with microtubule protein through 5 cycles of assemblydisassembly, co-sediments with microtubules in sucrose density gradients and, therefore, may be an integral part of the microtubule. Under non-polymerizing conditions, NDP kinase may be an integral part of multimeric microtubule rings (9,11).

While there is some evidence that microtubule associated NDP kinase catalyzes transphosphorylation at the tubulin E-site (7,9), this has not been satisfactorily established. As a first step toward studying the role of NDP kinase in transphorylation reactions, we have isolated the NDP kinase from bovine brain microtubule preparations. We have been able to isolate only very small amounts of this enzyme and so, as an aid to future studies, we have isolated the same NDP kinase directly from bovine brain cytosol. In this report, we document the properties of the enzyme from both sources.

EXPERIMENTAL PROCEDURES

Materials - Rabbit muscle pyruvate kinase, hog muscle lactate dehydrogenase, phosphoenolpyruvate, and ferritin were purchased from Boeringer Mannheim. $^{3}H_2O$, bovine serum [^{14}C] albumin (BSA[1]), and NEN-963 mixture were New England Nuclear products. SDS-PAGE standards were obtained from Bio-Rad and Boehringer Mannheim. All other chemicals for electrophoresis were obtained from Bio-Rad. Blue Dextran 2000, Pharmalytes, Sephadex IEF, and all chromatography column components were Pharmacia products, except for Fractogel TSK-55 (superfine) which was purchased from Pierce. Most other reagents were purchased from the Sigma Chemical Company. [γ-^{32}P] ATP was synthesized by the method of Glynn and Chappel (12) as modified by Walsh et al. (13).

Preparation of Microtubule Protein - Microtubule protein was prepared by the method of Borisy et al. (14) as modified for use with bovine brain by Murphy and Hiebsch (15). In a typical preparation, 700 grams of bovine brain, placed on ice within 20 minutes of slaughter, was trimmed of blood clots and meninges and was homogenized in PMED buffer (1 ml/gram of tissue) with a Tekmar Tissuemizer. PMED buffer contained 0.1 M PIPES, pH 6.6, 1 mM $MgCl_2$, 1 mM EGTA, and 2 mM dithiothreitol. The homogenate was centrifuged in a Sorvall GSA rotor at

12,000 rpm for 30 minutes at 4°C. The supernatant was centrifuged in a Sorvall SS-34 rotor at 18,000 rpm for 45 minutes at 4°C. The supernatant fraction resulting from this second spin was warmed to 35°C, GTP was added to a final concentration on 1 mM, and the mixture was allowed to polymerize for 30 minutes. Microtubules were then pelleted by centrifuging in the SS-34 rotor at 18,000 rpm and 25°C for 50 minutes. The pellets were resuspended in 40 ml PMED at 4°C and recentrifuged at 18,000 rpm for 60 minutes at 4°C in the SS-34 rotor. The supernatant portion was removed and was warmed to 35°C, GTP was added to a final concentration of 1 mM, and polymerization was allowed to continue for 20 minutes.

Microtubules were harvested by centrifugation in the SS-34 rotor at 18,000 rpm for 50 minutes at 25°C. The pelleted microtubules were quickly frozen in a dry ice-methanol constant temperature bath and stored at -80°C. When required for experiments, these microtubule pellets were thawed to 4°C, resuspended in a buffer appropriate to the experiment, and centrifuged in the SS-34 rotor at 18,000 rpm for 60 minutes at 4°C. This starting material was designated "2 cycle microtubule protein" and was used in all the isolations of NDP kinase from microtubule protein discussed in this paper.

Preparation of Microtubule NDP Kinase - All steps in this isolation procedure were performed at 4°C. A carboxymethyl-Sepharose CL-6B column (3.6x18 cm) was equilibrated with MMD (50 mM MES, pH6.2, 1 mM $MgCl_2$, and 2 mM dithiothreitol). Microtubule protein in MMD was loaded onto the column and the column was washed in MMD. The NDP kinase bound to the column was eluted with 0.75 M NaCl in MMD. The NDP kinase containing fractions were pooled and concentrated to 3 ml with a Millipore CX-30 vacuum ultrafilter and loaded on to a Sephadex G-75 (fine) column (2.5 x 18.5 cm) which had been equilibrated in 50 mM Tris-HCl, pH 7.3, 2 mM dithiothreitol. NDP kinase was eluted with this same buffer. The NDP kinase containing fractions were pooled and loaded onto a small (1-5 ml) column of Blue Sepharose CL-6B which had been equilibrated in 50 mM Tris-HCl, pH 7.3, 2 mM dithiothreitol. The column was washed in the same buffer and NDP kinase was eluted in 2 mM ATP and 2 mM $MgCl_2$. In some preparations a linear gradient of ATP and $MgCl_2$ was used to elute the enzyme from Blue Sepharose. NDP kinase containing fractions were pooled, concentrationed with a CX-30 ultrafilter, and chromatographed on a column of TSK-55 (3.2x38 cm) in the same Tris-dithiothreitol buffer. NDP kinase fractions from TSK chromatography were then chromatofocused on a PBE-94 chromatofocusing column (Pharmacia) with a 9.0-6.0 pH gradient. The start buffer was 50 mM ethanolamine which had been titrated to

pH 9.2 with acetic acid. The gradient was formed with Polybuffer 96 (Pharmacia) diluted 1 to 10, supplemented to 2 mM with dithiothreitol, and titrated to pH 6.0 with acetic acid. NDP kinase containing fractions were again concentrated with a CX-30 ultrafilter, frozen in a dry ice-methanol constant temperature bath and stored at -80^{o}C until use.

Isolation of NDP Kinase Directly from Brain - NDP kinase was isolated directly from brain by the same procedures used to isolate it from microtubule protein, with the addition of several preliminary steps. Bovine brain (1000-1600 grams) was obtained within 20 minutes of slaughter and immediatly placed on ice. All subsequent steps were performed at 4^{o}C. The brains were cleaned of blood clots, minced, and homogenized with a Tekmar Tissuemizer in 1 ml/gram of 0.1 M PIPES, pH6.62, 2 mM dithiothreitol. The homogenate was centrifuged in a Sorvall GSA rotor for 60 minutes at 10,000 rpm. We then performed a 10 % to 27 % polyethylene glycol-8000 (PEG-8000) cut on the supernatant. The supernatant was supplemented to 10 % PEG-8000 by adding an appropriate volume of 50 % PEG-8000 slowly and with vigorous stirring. After 20 minutes of stirring this was centrifuged in a GSA rotor at 10,000 rpm for 20 minutes. The supernatant of this spin was supplemented to 27 % PEG-8000 and after 30 minutes was centrifuged as before.

The supernatant of this spin was discarded and the pellets were frozen at -80°C until they were used. These pellets were resuspended in 200-300 ml of 50 mM MES, pH 6.2, 2 mM dithiothreitol by gentle Tekmar homogenization and the resuspended pellets were centrifuged in a GSA rotor at 12,000 rpm for 30 minutes. The supernatant of this spin was used as starting material for the further isolation of NDP kinase by the same procedure that had been used for the isolation of the microtubule protein preparation NDP kinase. This procedure consisted of 5 chromatography steps: CM-Sepharose, Sephadex G-75, Blue Sepharose, TSK-55, and chromatofocusing on PBE-94. For some preparations the Sephadex G-75 step was eliminated. The final product was stored at -80°C.

Electrophoresis - Discontinuous SDS-PAGE was done by the method of Laemmli (16) with some modifications when the gels were to be silver stained. For silver staining the gels were 0.75 mm gradient gels of 9-16 % acrylamide with a bis-acrylamide to acrylamide ratio of 0.053 which is twice the usual ratio. The gel solutions were passed through a 0.2 uM Millipore filter before polymerization to remove insoluble material. Samples were prepared by placing in a boiling water bath for 2 minutes and mixing with two volumes of 0.25 M Tris HCl, pH 6.8, 5 % SDS, 3 % sucrose, and 10 mM dithiothreitol. They were placed

in a boiling water bath again for 3 minutes and when they had cooled to room temperature they were alkylated with 0.06 M iodoacetamide in the dark for 1 hour as suggested by Hashimoto et al. (17) in order to eliminate the artifact bands appearing on the stained gel. Immediatly after electrophoresis the gels were silverstained by the method of Merrill (18). This method was modified by adding two 15 minute water washes between the oxidizer and silver steps and a 1 minute water wash between the silver and developer steps and by using 5 % acetic acid to stop development.

Determination of Kinetic Constants - Kinetic data for both NDP kinases were analyzed using a computer program that performed a weighted non-linear regression-fitting of the data to the Michaelis-Menton equation as described by Duggleby (19). In all cases we assumed that the standard deviations of velocity measurements increased proportionatly with velocity. Nucleotide concentrations in all kinetic experiments were determined by optical density measurements at pH 7.0 using the following extinction coefficients (all in cm^2/umole): ϵ_{257}=14.7 for ATP, ϵ_{256}=12.4 for GTP, ϵ_{262}=10.0 for UTP, ϵ_{280}=12.8 for CTP, and ϵ_{267}=9.9 for dTDP.

Assays - NDP kinase was assayed by the pyruvate kinase-lactate dehydrogenase coupled enzyme system described by Bergmeyer (20). These assays were performed with 25-200 Nl of sample in a final volume of 500 Nl at 30°C. Adenylate kinase assays were done in the same way except that 1.6 mg/ml AMP replaced the dTDP. For each assay, one unit of activity is defined as the amount of enzyme causing the oxidation of 1 umole of NADH per minute. Protein content was determined by the method of Bradford (21) because polybuffers did not interfere with this assay.

Sedimentation Equilibrium Determination of Native Enzyme Molecular Weight - The molecular weight of NDP kinase was determined by a modification of the method of Pollet et al. (22) which we have developed. Protein samples were dissolved in 20 mM PIPES, pH 7.0, 2 mM dithiothreitol, 1 mM $MgCl_2$, 2 % PEG-4000, and 20 mg/ml Dextran T40 (Pharmacia). The Dextran T40 is added to form a stabilizing gradient so that minimal mixing occurs during rotor deceleration. The samples also contained 4 uCi of ^3H_2O per ml so that the volume of fractions removed after centrifugation could be measured by liquid scintillation counting. Protein samples of 150 ul were centrifuged in the Beckman Airfuge for 24 to 48 hours at 4°C. [^{14}C]-bovine serum albumin was included in every run as an internal standard since we did not have a

method for accuratly determining the angular velocity of the rotor. At the end of the centrifugation period, the tubes were carefully removed from the rotor and 10-30 Ml fractions were removed from the meniscus with a Beckman Microtubue Fractionator. Fractions were assayed for NDP kinase activity and then liquid scintillation counted for ^{3}H in a Beckman CPM-100 with NEN-963 mixture. Fractions from [^{14}C]-bovine serum albumin containing samples were counted for both ^{3}H and ^{14}C in the same way. The average radius from the center of rotation, r, of each fraction was read from a table created by a computer program that numerically integrated an expression for the volume V(r), between the meniscus and a horizontal plane through a point at a distance, r, from the axis of rotation[2] (22). When centrifuging at equilibrium,

$$(1) \qquad \frac{d(\ln C)}{dr^2} = \frac{M_r w^2 (1-Vp)}{2RT}$$

where C is the protein concentration; M_r is the molecular weight; V is the partial specific volume; w^2 is the angular velocity of the rotor; p is the density of the solution; T is the temperature; and R is the gas constant.

Thus, if we assume that $V_{(NDP\ kinase)} = V_{(BSA)}$, then:

$$(2) \quad \frac{\frac{d(\ln C)}{dr^2}_{(NDP\ kinase)}}{\frac{d(\ln C)}{dr^2}_{(BSA)}} = \frac{M_r(NDP\ kinase)}{M_r(BSA)} .$$

The quantity $d(\ln C)/dr^2$ was determined for each protein by least-squares fitting of ln C versus r^2 to a line where C is in counts/minute for BSA and in units/ml for NDP kinase. Equation 2 could then be solved for M_r(NDP kinase), the molecular weight of NDP kinase.

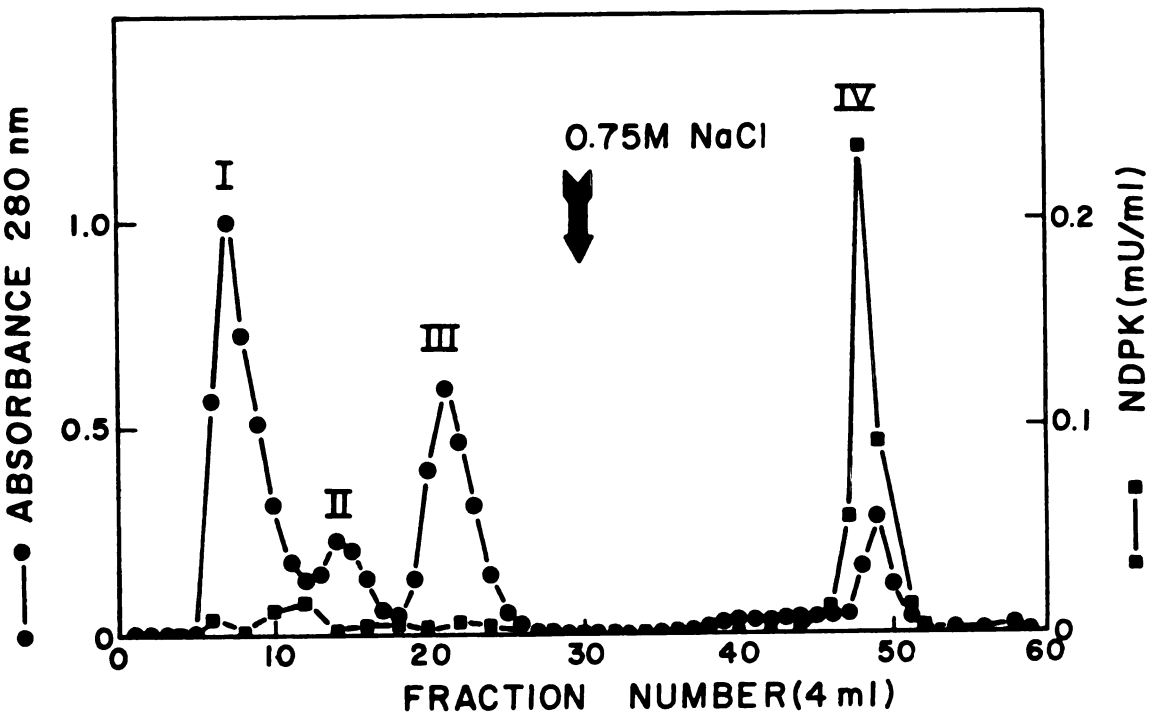
RESULTS

Isolation of Microtubule-associated NDP Kinase - We have been able to isolate the NDP kinase form 2 cycle microtubule protein by a 5-step procedure: CM-Sepharose, Sephadex G-75, Blue Sepharose, Fractogel TSK-55 (superfine), and chromatofocusing on PBE-94. This method produces a homogeneous preparation of NDP kinase but only in very small quantities. Using silver staining intensity to approximatly measure protein concentrations, we would estimate that from 1 gram of microtubule protein we can isolate only about 1-5 ng of NDP kinase, even though the yield of activity through the procedure is 15-20 %.

Figure 1 shows the chromatography of 2 cycle microtubule protein on CM-Sepharose. Both peaks I and II contained tubulin and high molecular weight proteins as shown by SDS-PAGE and, therefore, appear to represent different oligomers of tubulin and microtubule associated proteins. Peak III did not contain detectable protein and was probably nucleotide that was present in the microtubule protein starting material. Batch elution of the the column with 0.75 M NaCl yielded a NDP kinase preparation that was contaminated with adenylate kinase activity (1 unit/10 units of NDP kinase). Salt gradient elutions of NDP kinase were attempted, but NDP kinase eluted in three or more separate peaks. This might be

Figure 1. Carboxymethyl Sepharose chromatography of 2 cycle microtubule protein. Bovine brain microtubule protein (20 mg in 3 ml) was loaded on a column (3.6 x 18 cm) which had been pre-equilibrated in MMD at 4°C. The column was washed with MMD and then with 0.75 M NaCl in MMD while 4 ml fractions were collected. The absorbance of each fraction at 280 nm was measured and each fraction was assayed for NDP kinase activity. The NDP kinase peak eluted in 0.75 M NaCl contained 1.86 units of NDP kinase and 0.12 units of adenylate kinase activity.

Figure 1


ABSORBANCE 280 nm
NDPK(mU/ml)
0.75M NaCl
I
II
III
IV
1.0
0.5
0
0.2
0.1
0
0
10
20
30
40
50
60
FRACTION NUMBER(4 ml)

due to NDP kinase binding to other microtubule proteins or to the presence of multiple apparent isozymes.

The second step in the isolation procedure, chromatography on Sephadex G-75 (Figure 2), was performed in order to change the buffer and to remove the adenylate kinase activity which could not be separated from the preparation by the subsequent Blue Sepharose chromatography step in the procedure. Sephadex G-75-purified NDP kinase was then chromatographed on Blue Sepharose, which has been used to isolate a number of nucleotide requiring enzymes. NDP kinase bound to Blue Sepharose and could be eluted by ATP; either 2 mM ATP in a batch elution or a linear 0-7 mM ATP gradient as shown in Figure 3. NDP kinase could not be eluted from Blue Sepharose with UTP or GTP, although both of these nucleotides serve as phosphate donors in the NDP kinase reaction. If 2 cycle microtubule protein was chromatographed directly on Blue Sepharose without a preceding ion-exchange step, the NDP kinase was not retained by the column material. There may be sufficient nucleotide in 2 cycle microtubule protein to prevent NDP kinase binding to Blue Sepharose.

NDP kinase chromatofocused as a single peak or activity at a pH of 8.0 (Figure 4A). As we will discuss below, the NDP kinase sample for chromatofocusing was first chromatographed on Fractogel TSK-55, which removed ATP from the Blue sepharose elution buffer, and was then

Figure 2. Sephadex G-75 chromatography of Carboxymethyl Sepharose isolated NDP kinase. The NDP kinase fractions eluted from a Carboxymethyl Sepharose column as shown in Figure 1 were concentrated to 3 ml with a Millipore CX-10 ultrafilter and loaded on a 2.5 x 18.5 cm column of Sephadex G-75 fine. The column had been equilibrated and was developed with 50 mM Tris-HCl, pH 7.3, containing 2 mM dithiothreitol at 4oC. Fractions of 3 ml were collected and assayed for absorbancy at 280 nm, NDP kinase activity, and adenylate kinase (AK) activity. This step was included in the isolation procedure to desalt the preparation and to remove adenylate kinase.

Figure 2

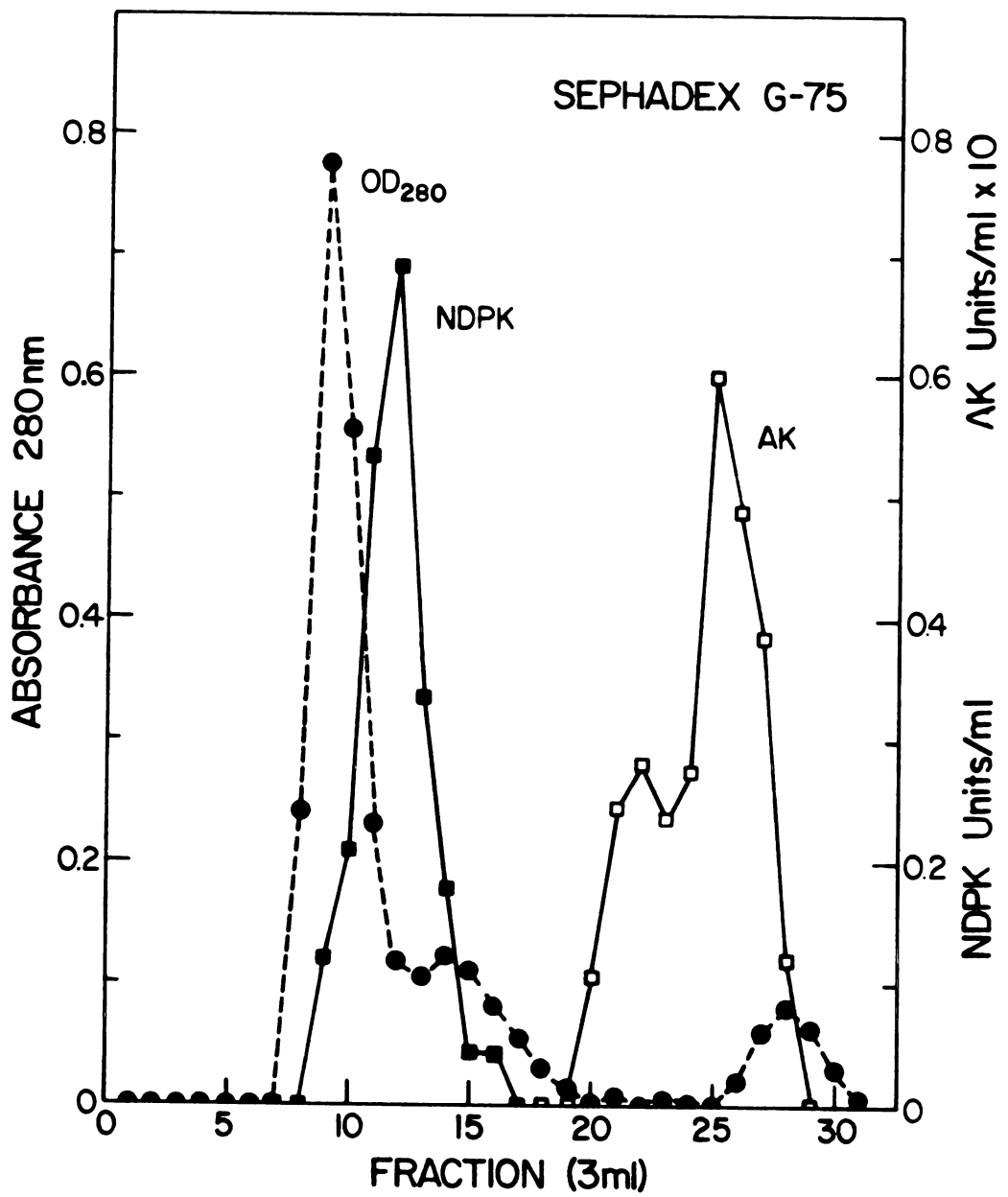

SEPHADEX G-75
OD280
NDPK
AK
ABSORBANCE 280nm
AK Units/ml x 10
NDPK Units/ml
FRACTION (3ml)
0
0.2
0.4
0.6
0.8
5
10
15
20
25
30

Figure 3. Blue Sepharose chromatography of Sephadex G-75 purified NDP kinase. Sephadex G-75 fractions containing NDP kinase activity were pooled and loaded on a 1 ml column of Blue Sepharose CL-6B which had been equilibrated in 50 mM Tris-HCl, pH 7.3, containing 2 mM dithiothreitol at 4oC. The column was washed first with 60 ml of the equilibration buffer and then with a 0-7 mM linear gradient of ATP in the same buffer. Fractions of 3 ml were collected and assayed for NDP kinase activity. Activity containing fractions were pooled, concentrated to 2 ml with a CX-10, and they were either stored at -60oC or used immediatly for the chromatofocusing step in the isolation procedure.

Figure 3

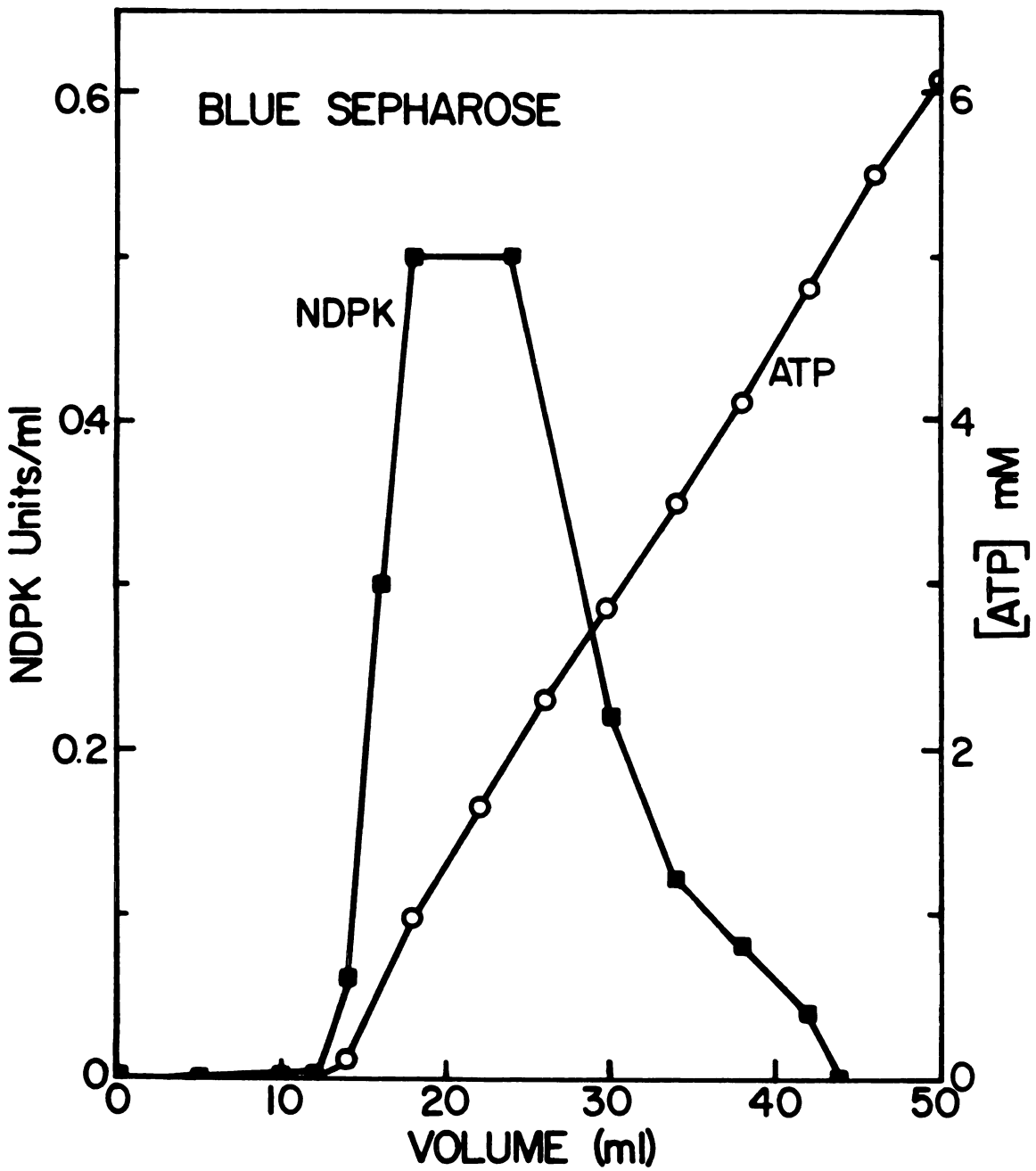

BLUE SEPHAROSE
NDPK
ATP
NDPK Units/ml
[ATP] mM
VOLUME (ml)
0.6
0.4
0.2
0
6
4
2
0
0
10
20
30
40
50

Figure 4. Chromatofocusing profiles of NDP kinase. Chromatofocusing was performed as described in "EXPERIMENTAL PROCEDURES".

A. Chromatofocusing of NDP kinase during its isolation from 2 cycle microtubule protein. The NDP kinase sample had previously been chromatographed on TSK-55s and had been supplemented to 0.6 mM with ADP 15 minutes before loading it on a 1 x 28 cm column of PBE 94.

B. Chromatofocusing of NDP kinase during its isolation directly from bovine brain. This step was performed just as described in part A.

C. Chromatofocusing of NDP kinase without a pretreatment with ADP during its isolation from microtubule protein. The NDP kinase sample had just been eluted from Blue Sepharose with 2 mM ATP and 2 mM $MgCl_2$. The ATP was not removed by a gel filtration step and the sample was not pretreated with ADP as in parts A and B. The sample was chromatofocused on a 1.2 x 30 cm column of PBE 94 which had been equilibrated at pH 8.4.

Figure 4A

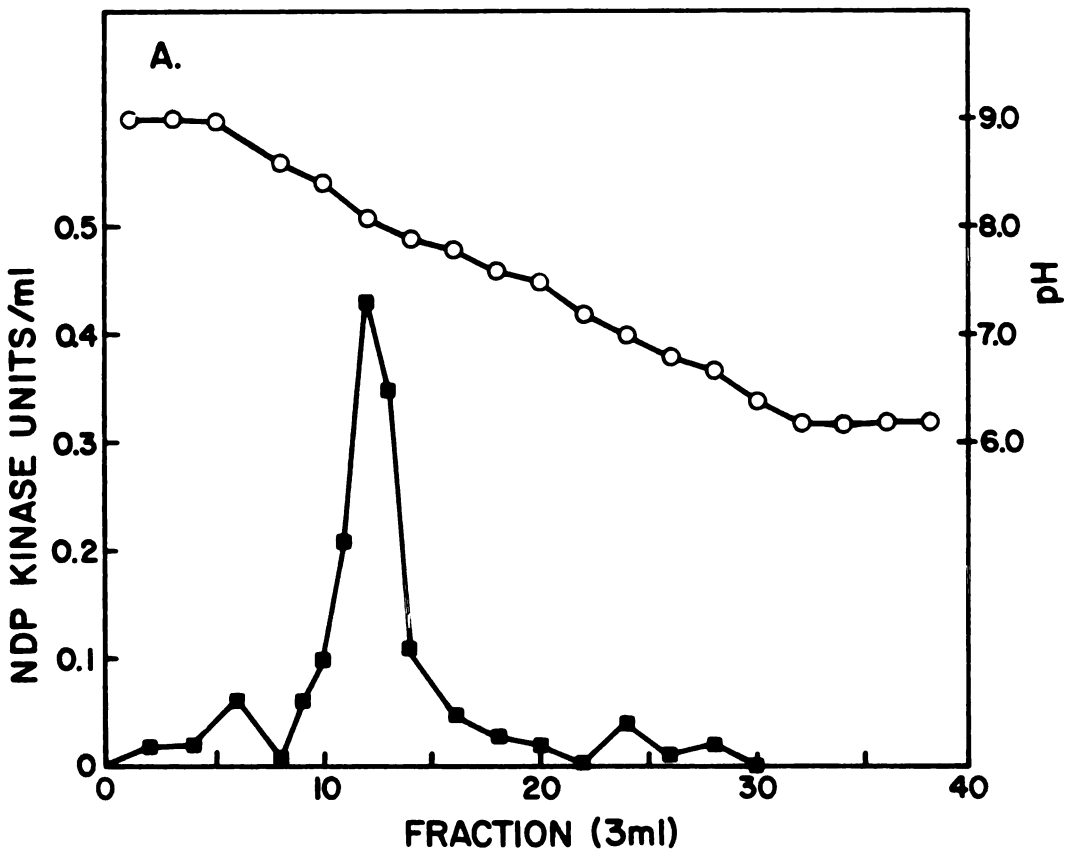

A.
NDP KINASE UNITS/ml
0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
FRACTION (3ml)
9.0
8.0
7.0
6.0
pH

Figure 4B

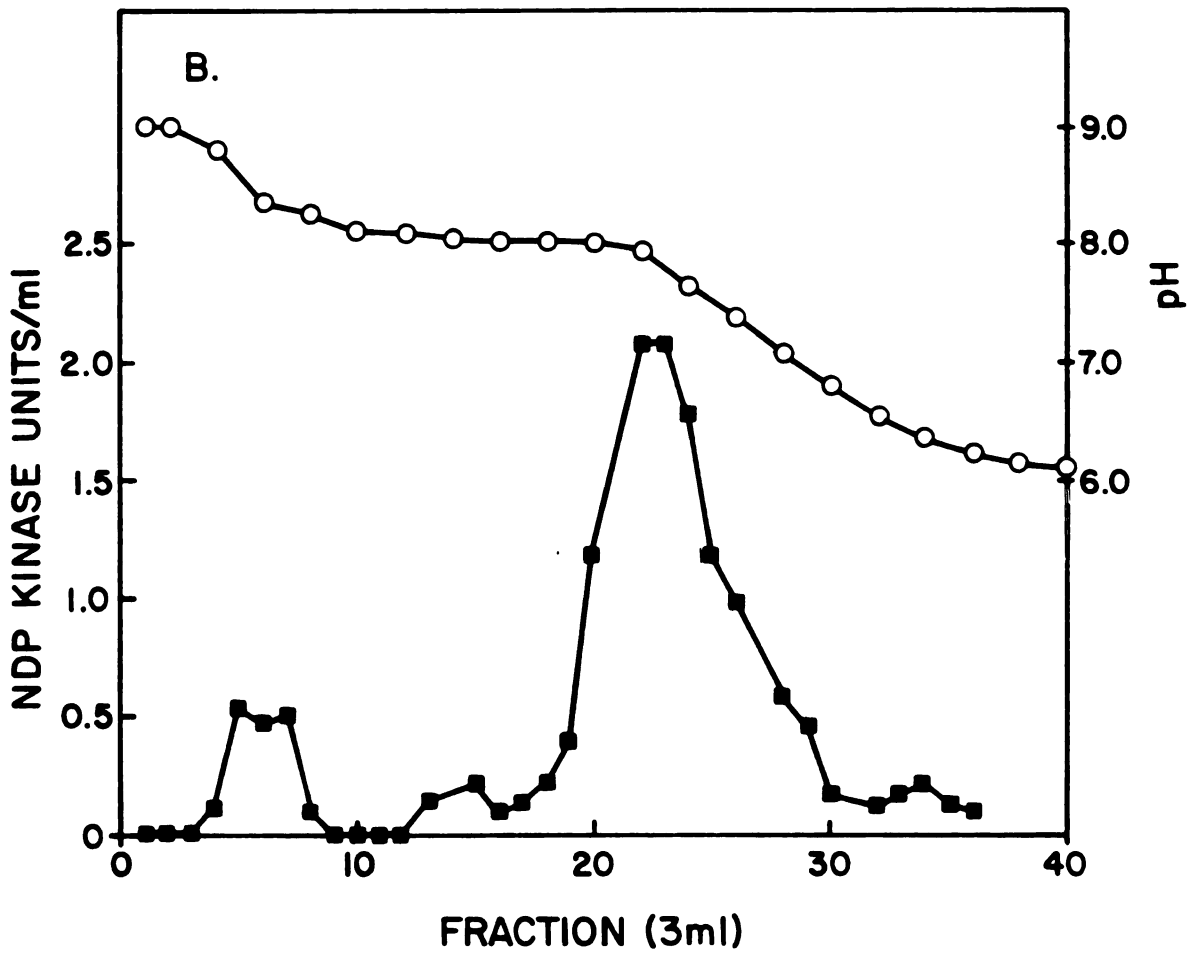
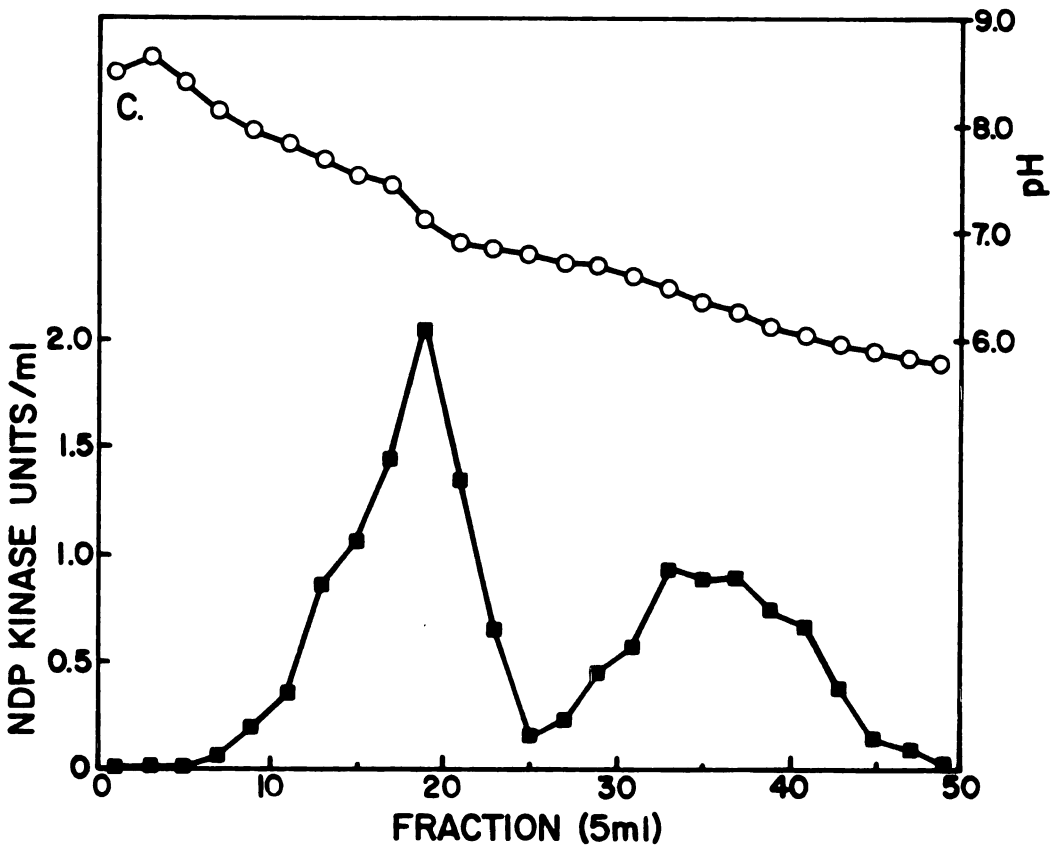

B.
NDP KINASE UNITS/ml
2.5
2.0
1.5
1.0
0.5
0
9.0
8.0
7.0
6.0
pH
0
10
20
30
40
FRACTION (3ml)

Figure 4C


C.
9.0
8.0
7.0
6.0
pH
2.0
1.5
1.0
0.5
0
NDP KINASE UNITS/ml
0
10
20
30
40
50
FRACTION (5ml)

treated with 2 mM ADP. This was done to remove the phosphate from any NDP kinase-phosphorylated intermediates that might be present and that might have a more negative charge than unphosphorylated NDP kinase.

This isolation procedure produced an NDP kinase that was free of detectable ATPase, GTPase, adenylate kinase, and protein kinase activities. The yield of NDP kinase from 2 cycle microtubule protein was 15-20%, but the -fold purification could not be determined since there was too little protein to measure.

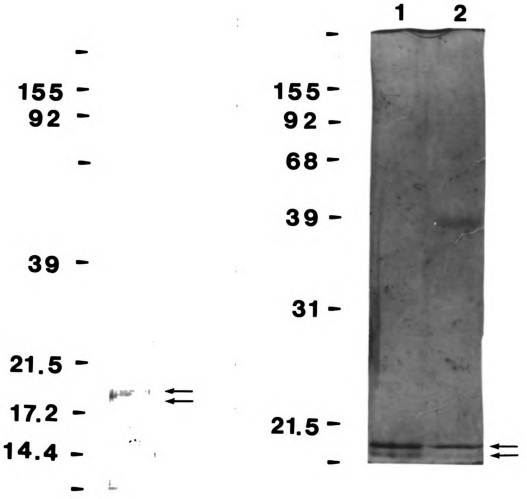
We have examined our isolated NDP kinase by SDS-PAGE. Coomassie Blue R-250 staining after electrophoresis was not sensitive enough to reveal protein bands, so gels were stained by the silver staining method of method of Merrill et al. (18). Several problems had to be overcome before protein bands could be detected by this procedure. Polybuffers in the final enzyme product interfered with the silver staining of NDP kinase in our initial gels which were 12% acrylamide. Polybuffers stained as diffuse yellow bands that obscured the lower molecular weight protein bands. For this reason, we decided to use 9-16% acrylamide gradient gels to seperate polybuffers as much as possible from protein bands. When the gels were fixed overnight in 10% acetic acid, 40% methanol, NDP kinase bands were not visible after staining. Hashimoto et al. (17) had discovered that proteins as large as 17,200 daltons were

Figure 5. Sodium dodeclysulfate polyacrylamide gel electrophoresis of NDP kinase. Electrophoresis and staining were done as described in "EXPERIMENTAL PROCEDURES". The standards used to estimate NDP kinase molecular weights were: lysozyme, 14,400; myoglobin, 17,200; soybean trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; RNA polymerase, 39,000; ovalbumin, 45,000; bovine serum albumin, 66,200; phosphorylase b, 92,500; and RNA polymerase, 155,000 and 165,000 daltons.

A. This gel shows the NDP kinase isolated from 2 cycle microtubule protein. After the initial silver staining this gel was cycled through silver nitrate and developer twice more. The background was lightened as described by Merrill et al. (18) and the gel was toned by the method of Berson (31). The gel was photographed with Polaroid type 55 P/N film and the negatives were printed on a variable contrast paper (Kodak Polycontrast Rapid II RC) at high contrast.

B. This gel shows both the NDP kinase isolated from microtubule protein (lane 1) and isolated directly from brain (lane 2). The preparations were run as far down the gel as possible so as to amplify any molecular weight differences that might exist. The gel was stained as for gel A.

Figure 5



eluted from their gels after soaking in similiar fixing solutions and that this elution was reduced by doubling the bisacrylamide to acrylamide ratio. Accordingly, we increased this ratio to 0.053 for our gradient gels and stained the gels immediatly after electrophoresis to minimize the elution of lower molecular weight proteins. A protein alkylation procedure (17) using iodoacetamide was performed on samples before electrophoresis to eliminate the artifact bands that frequently appear after silver staining. As shown in Figure 5, when using this electrophoresis and staining protocol, the NDP kinase isolated from 2 cycle microtubule protein was found to consist of a doublet of protein bands with apparent molecular masses of about 18,000 daltons. No other protein bands were visible.

Determination of Native Enzyme Molecular Weight - We could isolate only small amounts of NDP kinase from 2 cycle microtubule protein, and it lost activity as it became more dilute. Therefore, to determine the native enzyme molecular weight, we needed a technique that would alow us to use very small amounts of protein with a minimum of enzyme dilution. We chose a method developed by Pollet et al. (22) for sedimentation equilibrium determination of protein molecular weights using the Beckman Airfuge. We modified this technique as described in "EXPERIMENTAL PROCEDURES" for easier determination of

fraction volume and for determination of rotor velocity with a [^{14}C]-bovine serum albumin internal standard.

The results of such an experiment are shown in Figure 6. Figure 6A shows the sedimentation equilibrium profile plotted as NDP kinase activity vs. volume from the initial meniscus. r^2 was determined from the volume data with the use of a computer program and the data was replotted as shown in Figure 6B, we could see that ln C was a linear function of r^2, indicating that sedimentation equilibrium had in fact been achieved. Table I summarizes the results of three sedimentation equilibrium experiments with NDP kinase isolated from microtubule protein. The mean molecular weight of NDP kinase was 103,000 +/- 7,000. This result, along with the SDS-PAGE data discussed above, is consistant with an NDP kinase structure of six subunits, each of about 18,000 daltons.

Isolation of NDP Kinase Directly from Brain - We were able to isolate only very small quantities of NDP kinase from 2 cycle microtubule protein. We hoped to make an antibody to this enzyme and to do detailed molecular studies of NDP kinase function in microtubule events; however, these quantities were too small for these purposes. To obtain larger quantities of enzyme we decided to isolate it directly from bovine brain without a preliminary microtubule protein isolation. Most tissues

Figure 6. Sedimentation equilibrium profiles of NDP kinase isolated from microtubule protein. The experiment was performed as described in "EXPERIMENTAL PROCEDURES".

A. Samples (150 ul) of NDP kinase or [^{14}C] bovine serum albumin were centrifuged in the Airfuge at 4°C and 7 p.s.i. for 24 hours. At the end of the run 10 to 30 ul fractions were sucessively removed from the meniscus and assayed for NDP kinase activity or ^{14}C radioactivity. Each fraction was also assayed for $^{3}H_{2}O$ radioactivity to determine its volume.

B. In this figure the sedimentation equilibrium profile is plotted as ln C vs. r^2. The values of r^2 were determined from the volume between each fraction and the meniscus by a computer program as described in the text. From the ratio of the slopes of the two lines, the molecular weight of NDP kinase can be calculated to be 104,200.

Figure 6A

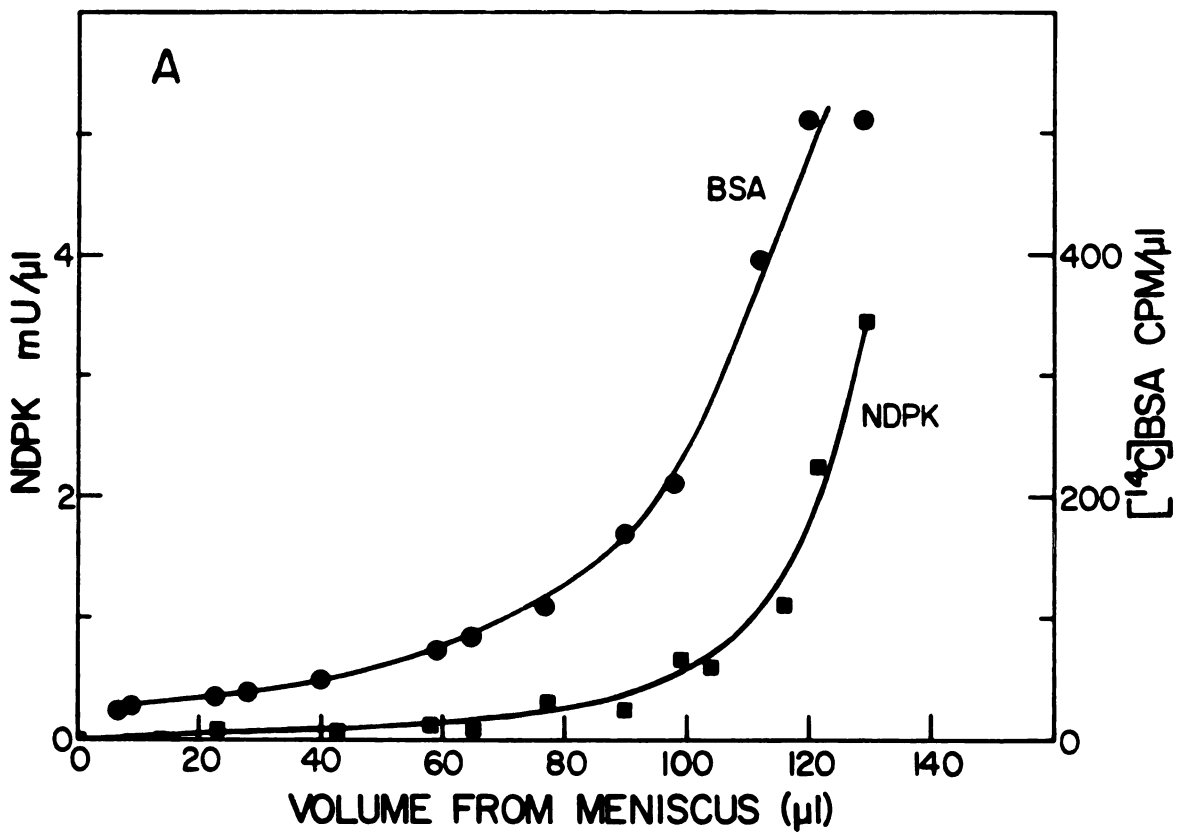

A
BSA
NDPK
NDPK mU/µl
[14C]BSA CPM/µl
VOLUME FROM MENISCUS (µl)
0
2
4
0
200
400
0
20
40
60
80
100
120
140

Figure 6B

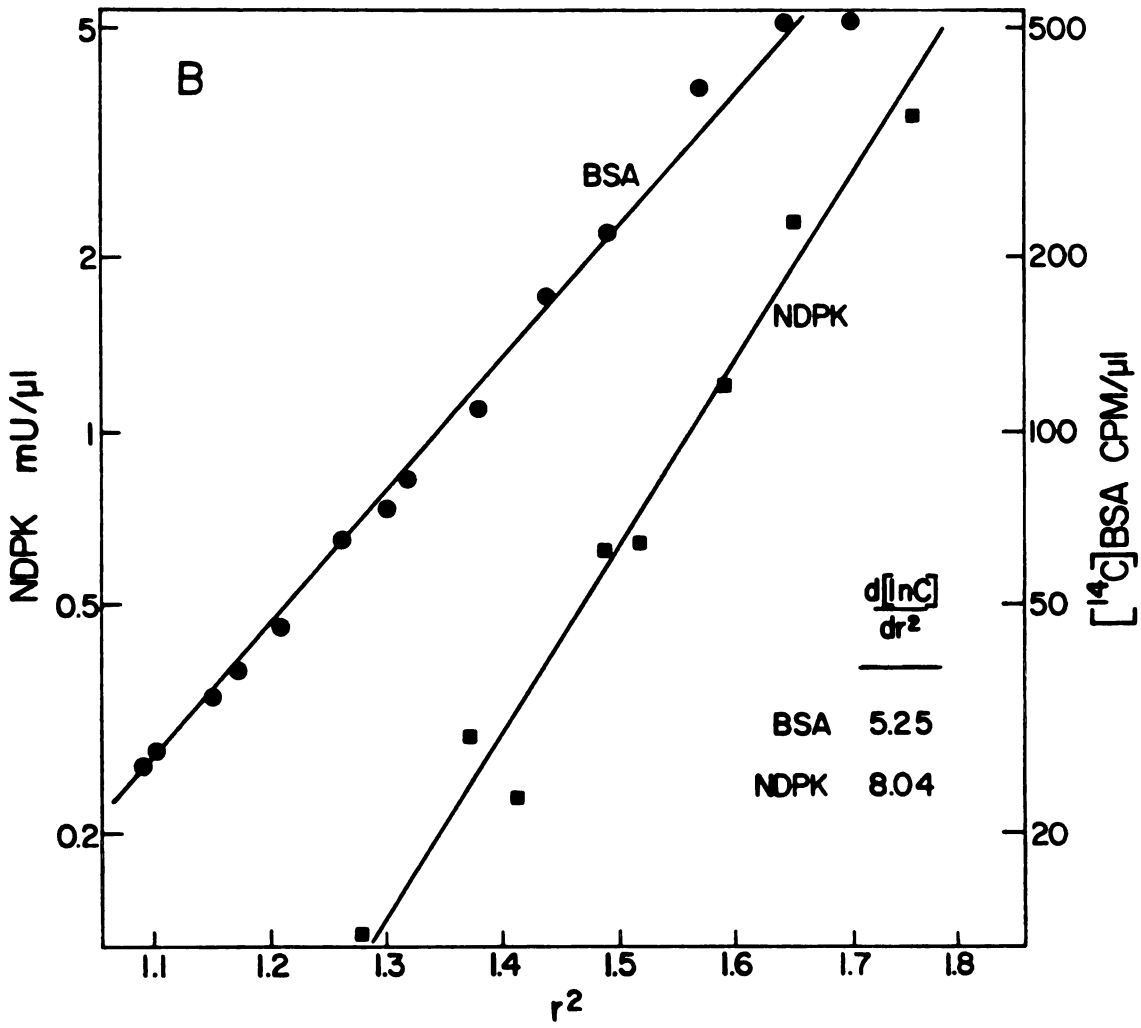

B
BSA
NDPK
NDPK mU/µl
[14C]BSA CPM/µl
r2
d[lnC]/dr2
BSA 5.25
NDPK 8.04
5
2
1
0.5
0.2
500
200
100
50
20
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8

TABLE I

Sedimentation equilibrium determination of NDP kinase molecular weight

This is a summary of the results of three sedimentation equilibrium determinations of the molecular weight of the NDP kinase isolated form microtubule protein. All three experiments were performed as described in "EXPERIMENTAL PROCEDURES". Experiment 3 is depicted in Figure 6.

Experiment	Time	Airfuge pressure	Approximate velocity	Molecular weight
	h	p.s.i.	rpm	
1	48	6	43,000	110,000
2	48	6	43,000	96,000
3	24	7	46,000	104,000
Mean +/- SE				103,200 +/- 6,800

have been reported to contain multiple isozymes of NDP kinase so, to avoid confusion, we decided to isolate NDP kinase from whole-brain by the same chromatographic procedures used to isolate the enzyme from microtubule protein preparations. In this way, if multiple forms were found at any step, then we would know which form to choose for further isolation. We needed a preliminary isolation step to decrease the volumes and the amount of protein. With this goal in mind, we produced a PEG-8000 precipitation profile of the NDP kinase from microtubule protein (data not shown) and found that this NDP kinase precipitated between 10 and 27% (w/v) PEG-8000. Accordingly we used this same 10 to 27% PEG-8000 differential precipitation step before the chromatography steps in our isolation of NDP kinase directly from brain.

A typical isolation of NDP kinase from brain is summarized in Table II. The procedure consisted of a 10-27% PEG-8000 fractionation, CM-Sepharose chromatography, dialysis, Blue Sepharose and Fractogel TSK-55 chromatography, and chromatofocusing from pH 9 to 6. Most of the NDP kinase in bovine brain was particulate and was pelleted in the first centrifugation step. Because the enzyme we were trying to isolate was cytosolic and not organellar, we used the first supernatant as a reference for calculating yield and -fold purification. The losses in NDP kinase yield

TABLE II

Isolation of NDP kinase directly from bovine brain

The isolation and assays were performed as described in "Experimental Procedures".

Step	Protein	Specific Activity	Purification	Yield
	mg	units/mg	-fold	%
Supernatant	29,400	0.11	1.0	100
10% PEG	17,000	0.30	2.7	85.9
27% PEG	3,800	0.30	2.7	32.0
CM-Sepharose	750	0.41	3.7	8.6
Blue Sepharose	11	14.3	130.0	4.5
TSK-55	3.0	37.7	343.0	3.2
Chromatofocusing	0.31	170.	1542.0	1.5

between the 10 and 27 % PEG-8000 steps probably represent storage losses of activity. The enzyme was routinely stored at -80oC when it was in the 27 % PEG-8000 precipitation pellet. NDP kinase was typically isolated directly from brain with an overall yield of 1-5 % and with a purification of 1500 to 2000-fold.

The chromatofocusing step in the isolation is shown in Figure 4B. NDP kinase focused at a pH of 8, the same as for the NDP kinase isolated from microtubule protein. In prelimianary attempts at isolationfrom both microtubule protein and directly from brain, chromatofocusing was done without a preceeding gel filtration step and without an incubation with ADP. A typical result is shown in Figure 4C. NDP kinase focused in multipke peaks at pHs below 8. NDP kinase from other sources have been reported to with phosphorylated intermediates (23-25). An NDP kinase hexamer could contain as many as six phosphates per molecule and the various phosphorylated forms would focus at more acidic pHs. By gel filtration on Fractogel TSK-55, we removed from the preparation the ATP, which had been used to elute the NDP kinase from Blue Sepharose. We could then remove the phosphate from the intermediate by incubating the enzyme with excess ADP with the result that the enzyme then focused as a single peak at a pH of 8 (Figures 4A and 4B).

SDS-PAGE of whole-brain NDP kinase showed that this enzyme, like the microtubule protein enzyme, consisted of two proteins of about 18,000 daltons. As shown in Figure 5B, these are the same subunit molecular masses present in the NDP kinase from microtubule protein. NDP kinase isolated directly from brain sometimes, but not consistantly, had a contaminant polypeptide band at 36,000-38,000 daltons (Figure 5B). This may be a dimer of NDP kinase subunits formed during sample processing.

Comparisons of the Two NDP Kinases - Both NDP kinases, isolated from microtubule protein and isolated directly from bovine brain, appeared to be identical. They were isolated by the same method and chromatographed in the same positions during all steps of the isolation procedure. Both preparations appeared identical on an SDS-PAGE gel (Figure 5B) and they had the same pI (Figure 4). To further test the hypothesis that the two NDP kinases were identical, we did two more experiments.

First, we measured the kinetic constants, Km and relative Vmax, for the two enzymes. As shown in Table III, the kinetic constants measured for both enzymes were similiar. The Km values for the two NDP kinases that we had prepared were very different from those reported by Robinson et al. (26) for the bovine brain particulate NDP kinase. This result is consistant

TABLE III

Comparison of kinetic properties of NDP kinases

Assays and calculations were done as described under "Experimental Procedures". Vmax values are reported as relative Vmax values which are the Vmax values relative to the Vmax for ATP for each enzyme.

Substrate	Microtubule enzyme[a]		Cytosol enzyme[b]		Particulate enzyme[c]	
	Km,app	Vmax	Km,app	Vmax	Km,app	Vmax
	mM	units	mM	units	mM	units
ATP	1.7	100	1.2	100	0.26	100
GTP	0.19	34	0.21	37	0.06	46
UTP		16[d]		6.3[d]	0.25	2.3
CTP		7.9[d]		5.0[d]	0.33	1.1
dTDP	0.57		0.58		0.26	

[a]This is the NDP kinase isolated from bovine brain 2 cycle microtubule protein preparations as described under "Experimental Procedures".

[b]This is the NDP kinase isolated directly from bovine brain cytosol as described under "Experimental Procedures".

[c]These are the values reported by Robinson et al. (26).

[d]Measured at a nucleotide concentration of 10 mM.

with the view that the NDP kinase isolated from microtubule protein preparations and the whole-brain NDP kinase are identical but that both are different from the brain particulate enzyme.

Next, we compared the thermal denaturation characteristics of the two NDP kinase that we had isolated. Both enzymes were heated at 60°C for various times in the presence of 1 mg/ml bovine serum albumin. The rates of activity loss were measured (Figure 7) and were the same for both enzymes. For both enzymes, 50% of the initial activity was lost in 9 minutes and 75% was lost in 19 minutes. This result is consistant with the idea that both enzymes are identical.

Phosphorylation of NDP Kinase - The behavior of NDP kinase on chromatofocusing columns (Figure 4) suggusted that this enzyme could be phosphorylated, changing its ionic properties, and that this phosphorylation was readily reversable, covalently bound phosphate being removed by ADP. We initially thought that the faster-migrating subunit observed after SDS-PAGE might be a phosphorylated form of the slower-migrating subunit. To test this view, we attempted to phosphorylate NDP kinase with [-32P]ATP. The enzyme was then electrophoresed in SDS and an autoradiograph of the dried gel was prepared. As shown in lane A of Figure 8, both subunit bands were phosphorylated. When NDP kinase that

had been phosphorylated in this way was subsequently treated with a 10-fold excess of ADP, most of the phosphate label was removed. This is shown in lane B of Figure 8 and suggests that the phosphorylated enzyme is an intermediate in the reaction, and not a more stable phosphate ester.

Figure 7. Heat denaturation of NDP kinase. Samples of NDP kinase isolated either from microtubule protein prearations or directly from bovine brain as described under "Experimental Procedures" was incubated in 0.1 M PIPES, pH 6.6, with 2 mg/ml bovine serum albumin in a 60°C water bath for various times. The incubation was terminated by placing the samples on ice. —●— ; the microtubule protein NDP kinase. —○— ; the NDP kinase isolated directly from bovine brain.

Figure 7

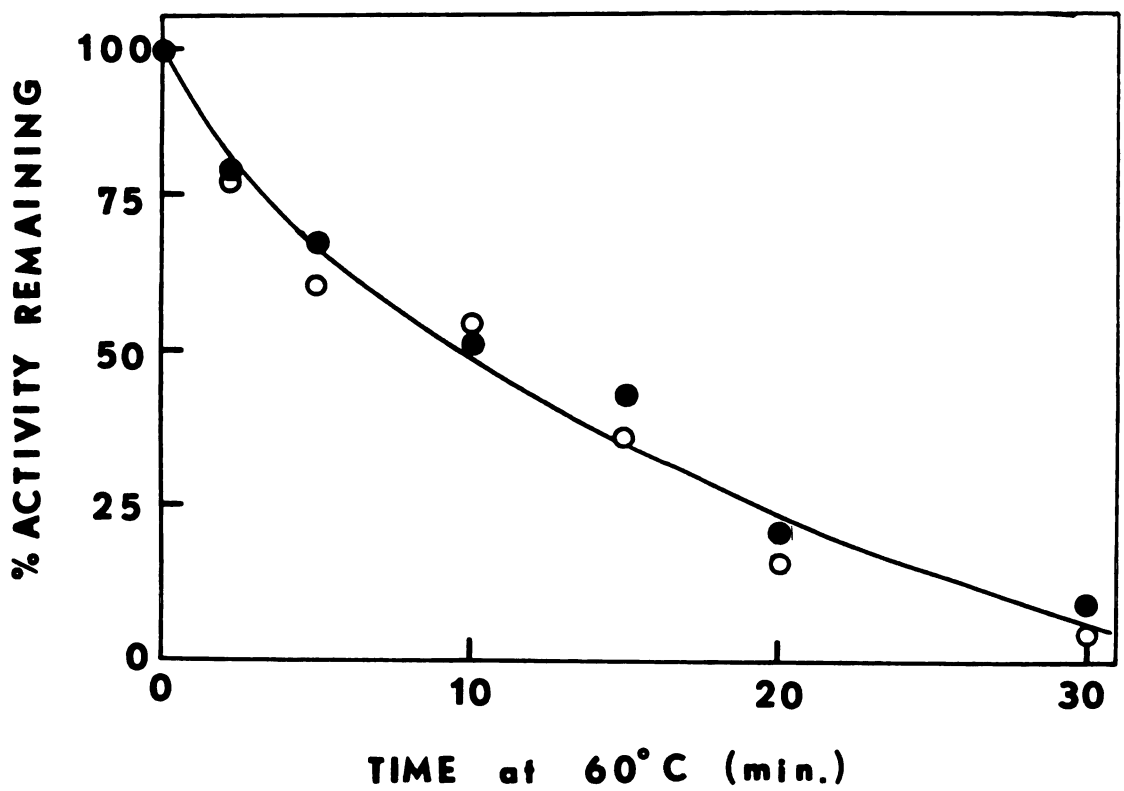
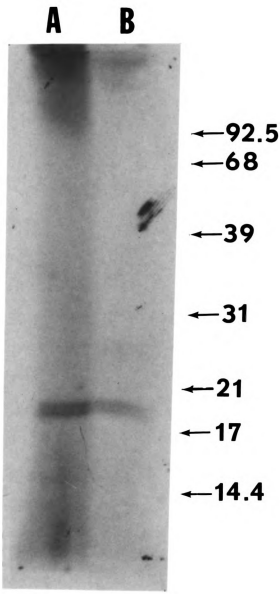


Figure 8. Autoradiograph of ^{32}P-labeled NDP kinase. Samples of NDP kinase isolated directly from bovine brain as described under "Experimental Procedures" (115 ng in 5 ul) were incubated with 5 uCi of [gamma-^{32}P]ATP (1100 cpm/pmole) in a total volume of 10 ul for 2 minutes at room temperature. The sample for lane A was mixed with 25 ul of SDS-PAGE sample buffer which contained 5% SDS, and then with 10 ul of ADP. The sample for lane B was mixed with 10 ul of ADP, incubated for 5 minutes at room temperature, and then mixed with 25 ul of SDS-PAGE sample buffer. Two lanes of standards; the same as shown in Figure 5; were run on the same gel. After electrophoresis, the two radioactive lanes were separated, dried onto filter paper, and autoradiographs were prepared. The lanes of standards were fixed in 12.5% (w/v) trichloroacctic acid, stained with 0.1% (w/v) Serva blue W overnight, and destained in water. A doublet of bands was phosphorylated with molecular weights of about 18,000. As can be seen by comparing the two lanes, the 5 minute chase with nonradioactive ADP removed much of the labeled phosphate for the sample in lane B.

Figure 8



DISCUSSION

We have isolated the NDP kinase from 2 cycle microtubule protein in relatively high yield but only in nanogram quantities. This enzyme has a molecular mass of 103,000 +/- 7,000 daltons as determined by sedimentation equilibrium ezperiments (Table I). On silver stained SDS-PAGE gels, this NDP kinase has a doublet of protein bands at about 18,000 daltons (Figure 5). We therefore conclude that microtubule protein NDP kinase is a hexamer, but we cannot determine the exact composition of subunits. This microtubule protein NDP kinase chromatofocused as a single isozyme with a pI of 8.0 after removal of ATP and dephosphorylation with ADP (Figure 4).

In order to obtain larger quantities of this enzyme, we isolated it directly from brain without a preliminary microtubule protein isolation. To insure that we would isolate the same isozyme of NDP kinase directly from brain that we had isolated from 2 cycle microtubule protein, we used an identical isolation procedure with the addition of a 10-27% PEG-8000 differential precipitation (Table II). This whole-brain NDP kinase chromatofocused at a pH of 8.0 (Figure 4) and contained at least two different subunits of about 18,000 daltons. We think that both of the enzymes we have isolated, the one isolated from microtubule protein and the one

isolated directly from brain, are identical. They have the same pIs. They are isolated by the same chromatographic procedures and behave similiarly during each chromatographic step. The two enzymes have similiar catalytic behavior, similiar Km values and relative Vmax values, and they thermally denature at the same rates. We propose that they are the same enzyme.

Robinson et al. (26) have isolated a NDP kinase from the particulate fraction of bovine brain. This NDP kinase was a hexamer of 16,600 dalton subunits and did not consist of two separate subunits as revealed by SDS-PAGE in contrast to the NDP kinase isolated in this study. The particulate enzyme had a pI of 8.4, similiar to the microtubule protein enzyme. The particulate enzyme could be eluted from Blue Sepharose by GTP, while under similiar conditions, the microtubule protein enzyme could not, even though it would use GTP for a substrate. The particulate and microtubule protein NDP kinases had different catalytic properties (Table III). Finally, the microtubule enzyme would not compete with detergent micelles for Blue Sepharose dye binding as would the particulate form and could, therefore, not be isolated by the method of Robinson et al. (26). The NDP kinases that have been studied have been principally cytoplasmic or mitochondrial enzymes (27) although, in rat liver, 12% of the total NDP kinase activity is nuclear (28). Karr et al. (29) have presented convincing evidence that the NDP

kinase of bovine brain microtubule protein preparations is not of mitochondrial origin. We suggest that the particulate NDP kinase from bovine brain and the NDP kinase that we have isolated in this study are different.

There are several possible explanations for the two subunit bands present after SDS-PAGE of NDP kinase from microtubule protein peparations. One is that they are different gene products. A second possibility is that they are created by some sort of post-translational modification of a single gene product. At first, we thought that the faster-running species might be a phosphorylated intermediate, but this can be ruled out, since, as seen in Figure 8, both bands are phosphorylated. We cannot, however, discount the possibility that the faster running species is phosphorylated creating a stable phosphate ester, not at the active site. Robinson et al. (26) found that treatmentof the bovine brain particulate NDP kinase with various proteases produced both a loss of activity and the appearance of multiple isozymes, electrofocusing at lower pHs. Although our NDP kinase was a single isozyme, it is still possible that removing a few amino acid residues by protease action could produce the lower molecular weight form without substantially altering the enzymes pI.

The NDP kinase isolated directly from brain could be phosphorylated to a high-energy phosphate intermediate as shown in Figure 8 which could be dephosphorylated by the addition of ADP. Both subunits could be phosphorylated, suggesting that they are both catalytic subunits. We believe that many of the multiple forms of NDP kinase that we encountered during early attempts at isolation were caused by this phosphorylation. For example, when NDP kinase was chromatofocused without the removal of ATP and pretreatment with ADP, multiple, more acidic forms of the enzyme were observed (Figure 4C). In retrospect, this is not surprising; as many as six phosphates could be present on any one NDP kinase molecule and this would substantially change its charge properties.

It has been suggested by Penningroth and Kirschner (7) that a microtubule-associated NDP kinase might catalyze the transphosphorylation of tubulin E-site GDP to GTP without this nucleotide leaving its binding site. This guanine nucleotide is tightly bound at the E-site when tubulin is in oligomeric microtubule protein rings (8) and may not freely exchange with GTP. Of course, it is still not known whether such rings exist <u>in vivo</u>, but they or some similiar form of microtubule protein oligomer may exist in the cell and require NDP kinase to regenerate E-site GTP. In this way, NDP kinase could control microtubule assembly or function. The idea that NDP kinase could regulate a GTP-dependent process is not

unprecedented. The activity of plasma membrane guanine nucleotide-dependent adenylate cyclase may be regulated by the binding of NDP kinase to nearby sites in the plasma membrane (30). Now that we have isolated the NDP kinase from microtubule protein preparations we have a tool for studying the role that this enzyme may play in microtubule assembly and function.

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CHAPTER IV

THE ASSOCIATION WITH MICROTUBULES OF A NUCLEOSIDE DIPHOSHATE KINASE HAVING TRANSPHOSPHORYLASE ACTIVITY

INTRODUCTION

Nucleoside diphosphate kinase (NDP kinase) activity has been found in preparations of brain microtubule protein isolated by cycles of assembly-dissassembly (1-3). It has been shown that this NDP kinase is not of mitochondrial origin, not released from organelles by homogenization, and, therefore, it is available for interaction with microtubules *in vivo* (4). NDP kinase has also been found in proximity to the microtubules of flagella in sea urchin sperm (5) and Chlamydomonas (6) and to the microtubules of Tetrahymena cilia (7).

The tubulin dimer contains two distinguishable guanine nucleotide binding sites (8-11). At one of these sites called the exchangeable site (E-site) the nucleotide must usually be GTP in order to support microtubule assembly and this GTP is hydrolyzed to GDP as assembly proceeds (12-14). There are at least two mechanisms by which E-site GTP may be regenerated so that the dimer may participate in further assembly reactions: transphosphorylation and nucleotide exchange.

Exchange:

$$\text{Tubulin-GDP} + {}^{*}\text{GTP} \rightleftharpoons \text{Tubulin-}{}^{*}\text{GTP} + \text{GDP}$$

Transphosphorylation:

$$\text{Tubulin-GDP} + {}^{*}\text{GTP} \rightleftharpoons \text{Tubulin-GTP} + {}^{*}\text{GDP}$$

It has been proposed that NDP kinase may catalyze the proposed transphosphorylation reaction by which GDP bound to the tubulin E-site may be phosphorylated without ever leaving that site (1,2). If this is true, then NDP kinase might play an important regulatory role in the regulation of microtubule assembly and function. The work of Penningroth and Kirschner (1) showed that when microtubule protein was incubated with [^{3}H] GTP and [gamma-^{32}P] GTP under conditions that would support microtubule assembly, ^{32}P labelled nucleotide appeared on the tubulin dimer almost 10 times faster than did ^{3}H labeled nucleotide. Thus, they concluded that transphosphorylation is occuring 10 times faster than simple nucleotide exchange. A similiar experiment and result has been reported by Jacobs and Huitorel (2) but the validity of these experiments has been challenged by Brylawski and Caplow (15) who have suggested that the results are artifactually caused by the presence of pools of free nucleotide that are difficult to remove.

As a result of these studies we believe that there are important questions to be answered about NDP kinase and microtubules. We want to know whether there is really a transphorylation mechanism for the generation of E-site GTP and whether that reaction is catalyzed by NDP kinase? Next, we want to know whether NDP kinase binds to microtubules and to microtubule protein oligomers both *in vitro* and in the cell.

In recent studies (16) we have isolated and characterized the NDP kinase from bovine brain microtubule protein. Most of our findings about this NDP kinase have been confirmed for the porcine brain microtubule NDP kinase by the subsequent work of Huitorel *et al*. (17). In this paper we will begin answering questions about the role of NDP kinase in microtubule function and about the possible binding of NDP kinase to microtubules and microtubule protein oligomers and we will be discussing the development of techniques and strategies that will permit their ultimate resolution .

EXPERIMENTAL PROCEDURES

Materials - Rabbit muscle pyruvate kinase, hog muscle lactate dehydrogenase, phosphoenolpyruvate, and ferritin were purchased from Boeringer Mannheim. Bovine serum [^{14}C] albumin (BSA1) and NEN-963 liquid scintillation mixture were New England Nuclear products. SDS-PAGE standards were obtained from Bio-Rad and Boehringer Mannheim. All other chemicals for electrophoresis were obtained from Bio-Rad except for Serva blue stains which were a product of Serva Fine Biochemicals. Blue Dextran 2000 and all chromatography column components were Pharmacia products, except for Fractogel TSK-55 (superfine) which was purchased from Pierce. Most other reagents were purchased from the Sigma Chemical Company. [gamma-^{32}P] ATP was synthesized by the method of Glynn and Chappel (18) as modified by Walsh et al. (19). Rabbit antiserum to tubulin and actin was purchased from Miles-Yeda. FITC-goat antirabbit IgG was obtained from Miles-Yeda and from Boeringer-Mannheim. Horse radish peroxidase coupled goat anti-rabbit IgG and peroxidase color development reagent were products of Bio-Rad and rabbit muscle actin was from Sigma. All solutions for tissue culture were obtained from Gibco.

Preparation of Microtubule Protein - Microtubule protein was prepared as previously described (16) except that the first assembly reaction was initiated by the addition of 2 mM ATP and 0.2 mM GTP instead of with 1 mM GTP.

Isolation of NDP kinase from Bovine Brain - NDP kinase was isolated from bovine brain cytosol by the method of Nickerson and Wells (16). Previous work has shown that this is the same NDP kinase present in bovine brain microtubule protein preparations (16).

The NDP kinase from the particulate fraction of bovine brain was isolated by the method of Robinson et al. (20) as modified in this laboratory. Bovine brain (1000 g) was homogenized in 1 ml/gram of tissue of 0.1 M PIPES, pH 6.62, 2 mM Dithiothreitol with a Tekmar Tissuemizer. This and all subsequent operations were done at 4°C. The homogenate was centrifuged at 12,000 rpm in a Sorvall GSA rotor for 1 hour. The pellets were extracted with 2 liters of 40 mM Tris HCl, pH 7.5, 10 % (v/v) ethanol, 2 % (w/v) BRIJ 56, 5 mM $MgSO_4$, and 1 mM EDTA. The pellets were resuspended in this buffer with the Tekmar Tissuemizer. The resuspended pellets were centrifuged as before for 2 hours. The supernatant fraction was mixed with 40 ml of swollen Blue Sepharose 6B and this mixture was gently agitated for 18 hours before filtering out the Blue Sepharose with a coarse

scintered glass funnel. The Blue Sepharose was poured into a column which was then washed with 1500 ml of 10 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$ before the enzyme was eluted with 250 mL of the same buffer containing 0.2 mM GTP. The eluate was concentrated to 7 ml with a Whatman CX-30 ultrafilter. Portions of this preparation (2 ml) were chromatographed on a 3.2 x 38 cm column of Fractogel TSK-55s in the same buffer. The NDP kinase containing fractions were concentrated with a CX-30 ultrafilter and frozen in aliquots at -80°C.

Assays - Protein concentrations were determined by the method of Lowry et al. (21) except during the isolation of brain cytosol NDP kinase when the method of Bradford (22) was used, because the polybuffers employed in the procedure interfered with the former assay. NDP kinase was assayed by coupled enzyme assay as previously described (16).

Electrophoresis Techniques - The identity and purity of protein preparations was determined by SDS-PAGE1 using the method of Laemmli et al. (23) except that in some experiments the gels used were 0.75 mm 9-16% (w/v) acrylamide gels with an acrylamide to bis-acrylamide ratio of 19. Gels were fixed in 15 % (w/v) trichloroacetic acid, washed twice with water, stained with 0.1 % (w/v) Serva Blue W, and destained in water.

Western blots were prepared by electrobotting samples from SDS-PAGE gels onto nitrocellulose membranes with a BioRad Transblot apparatus operated at 60 volts for 4 hours at 10°C. Electroblotting was performed in 25 mM Tris, 192 mM glycine, 10 % (v/v) methanol, and 0.01 % (w/v) sodium dodecyl sulfate. Gels were prepared for autoradiography by drying them onto Whatman 3MM paper at high temperature and under vacumn.

Immunochemical Techniques - Antibodies were raised in New Zealand white female rabbits. After preliminary bleedings to obtain pre-immune serum, rabbits were immunized with 500 ug of NDP kinase in Freunds complete adjuvant (Sigma). Injections were repeated at 10 day intervals with 500 ug of protein in Freunds incomplete adjuvant (Calbiochem) until antibody was detected. Thereafter, 150 ug injections in Freunds incomplete adjuvant were made at convenient intervals. Animals were bled from an ear vein 4 to 7 days after each injection. Serum was stored at -80°C.

The presence of antibody was detected by the method of Ouchterlony (24) except that the gels were 1 % agarose containing 25 mM Tricine, pH 8.6. Ouchterlony plates were stained by washing for several days against many changes of phosphate buffered saline, staining with 0.1 % Serva Blue W, and destaining with water.

The IgG fraction of rabbit serum was isolated on a 3 ml column of Protein A Sepharose. The column was equilibrated with 0.1 M MOPS, pH 7.0. Serum was passed through the column and it was washed with 20 ml of equilibration buffer. IgG was eluted with 0.1 M glycine, pH 3.1. Fractions of 1 ml were collected and immediatly neutralized by the addition of 75 ul of 1 M Tris base. The fractions containing eluted protein were pooled, dialyzed against 1 liter of phosphate buffered saline and concentrated with a Millipore CX-30 ultrafilter. Aliquots were frozen at -80oC.

NDP kinase was detected on nitrocellulose after electroblotting with the use of anti- NDP kinase IgG and the procedure of Towbin et al. (25). All steps were performed in Tris buffered saline (TBS: 20 mM Tris, pH 7.5, 500 mM NaCl), the blots were blocked in 3 % (w/v) gelatin in TBS, and the antibody incubations were done in 1 % (w/v) gelatin in TBS.

Nucleotide Affinity Labeling of Tubulin - The tubulin E-site was affinity labeled with periodate oxidized GDP by the method of Maccioni and Seeds (26). Periodate oxidized GTP was synthesized by a modification of the Maccioni and Seeds (26) method. One micromole of GTP, sometimes containing [3H] GTP, was brought to a final volume of 160 uL in water to which was added 20 ul of 50 mM sodium periodate (pH 5.5-6). The reaction was

allowed to proceed at 4°C for 30 minutes when 10 ul more of periodate was added for a further 30 minute incubation. Excess periodate was removed by adding 10 ul of 50 mM glycerol. The product, oxGTP, was isolated by a modification of the method of Palmer and Avruch (27). The reaction products were loaded on a 3 ml column of DEAE Sepharose which had been equilibrated in saturated sodium bicarbonate and then washed with 30 ml of water. oxGTP was eluted with a 0.1- 1.5 M linear gradient of triethylamine carbonate buffer. A 2 M stock solution of this buffer was made by bubbling CO_2 through 28 % (v/v) triethylamine until the pH decreased to 7.5. Nucleotides were detected by absorbancy at 260 nm. Peaks were pooled, frozen, and lyophylized. The purity of oxGTP was checked by thin layer chromatography on PEI-cellulose plates developed in 1.2 M LiCl.

Microtubule protein (2 cycle) was treated with Norit A charcoal (0.1 mg per mg of protein) which had been extensively washed with 2 % (w/v) bovine serum albumin and then with water. The microtubule protein was incubated at 30°C for 20 minutes and centrifuged for 15 minutes at 175,000 x g in the Beckman Airfuge. The microtubule pellets were resuspended in 10 mM Tris HCl, pH 6.8, 20 mM sodium borohydride and incubated for 30 minutes on ice before dialysis against 2x1 liter changes of 0.05 M PIPES, pH 6.62. Dialyzed protein was frozen at

-80°C in aliquots. This microtubule protein, because it has been through an assembly-disassembly cycle, is affinity labeled with oxGDP.

Tissue Culture and Immunofluorescent Staining - 3T3 mouse fibroblasts were obtained from Dr. John Wang of this department. They were cultured in Eagles Modified Essential Medium containing 100 ug/ml streptomycin, 100 U/ml penicillin, and 10 % (v/v) newborn calf serum at 37°C in a water saturated atmosphere of 10 % (v/v) CO_2. Cells were passed by treatment with 0.25 % (w/v) trypsin in phosphate buffered saline (PBS) at 35°C for 3 minutes and then with 0.025 % (w/v) EGTA[1] in PBS at 35°C for 20 minutes. For immunochemical localization studies, cells were grown on glass coverslips which had been washed in 100 % ethanol and then autoclaved.

Methods for immunofluorescent staining were adapted from the the procedures of Weber and Osborn (28). Cells grown on coverslips were washed for 10 seconds in PBS at 37°C and then fixed in pure methanol at -10°C for 6 minutes. The cells were rehydrated by immersion in PBS at room temperature for 30 seconds and were then ready for staining.

The coverslips were placed in a humidity chamber for incubations with antibody. They were coated with an appropriate dilution of rabbit anti-NDP kinase, anti-tubulin, or anti-actin or with the same dilution of

preimmune serum and incubated for 30-60 minutes. The coverslips were rinsed in 4-6 changes of PBS and covered with an appropriate dilution of FITC conjugated goat anti-rabbit IgG and incubated for 30-45 minutes. After a further 4-6 washes in PBS the coverslips were mounted on slides in a drop of mounting medium containing 10 % (v/v) 0.1 M sodium phosphate, pH 8.5, and 90 % (v/v) glycerol. They were then viewed and photographed with a Leitz Orthoplan fluorescence microscope with optics optimized for FITC fluorescence. Appropriate dilutions of all antibodies were chosen empirically and varied considerably from batch to batch of the same antibody.

RESULTS

The Association of NDP Kinase with Oligomeric Microtubule Protein Rings -Previous studies have suggested that most of the NDP kinase from microtubule protein preparations binds to multimeric microtubule protein rings under non-assembling conditions (2,29). This is consistent with a role for NDP kinase in transphosphorylation since ring E-site nucleotide is not readily exchangeable and might require transphosphorylation to regenerate GTP (2,30). This suggested that the association of NDP kinase with rings might be an important phenomena, so we attempted to confirm that NDP kinase was associated with rings. Oligomeric rings were separated from non-aggregated protein by chromatography on columns of Sepharose CL-6B. As shown in Figure 1B, only 16 % of the NDP kinase eluted with oligomeric rings in the void volume. Previous studies, which have shown a stronger association of NDP kinase with rings, had been done with high concentrations of glycerol in the buffers and done with microtubule protein that had been isolated in the presence of 6 M glycerol. When our experiment was repeated in the same buffer but this time containing 6 M glycerol, a much higher percentage of NDP kinase associated with rings (Figure 1C). The addition of 6 M glycerol to isolated NDP kinase did not cause isolated NDP kinase to aggregate and elute in the void volume

Figure 1. Binding of NDP kinase to oligomeric microtubule protein rings. One of two identical pellets of microtubule protein was resuspended in 50 mM MES, pH6.6, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol and the second was resuspended in 2.5 ml of the same buffer containing 4 M glycerol. The resuspended pellets were centrifuged at 18,000 rpm at 4°C in a Sorvall SS-34 rotor. Two 1 ml samples (4.4 mg/ml, 50 mU NDP kinase/mg) of the resuspended microtubule protein were chromatographed on similiar 2.8 x 17 cm columns of Sepharose 6B preequilibrated in the same buffer (part B) or in the same buffer at pH 6.2 (Part A). A 1 ml sample of 4 M glycerol resuspended microtubule protein (5.0 mg/ml, 55 mU NDP kinase/mg) was chromatographed on a similiar column that had been equilibrated in the same buffer containing 4 M glycerol (part C). Chromatography was performed at 4°C at a flow rate of 36 ml/hour and 3 ml fractions were collected, assayed for absorbancy at 280 nm, and assayed for NDP kinase activity. The column was calibrated with the following standards: Vo, Blue Dextran 2000 (2,000 kilodaltons); thyroglobulin (670 kdaltons); ferritin (480 kdaltons); gamma-globulin (158 kdaltons); ovalbumin (44 kdaltons); myoglobin (17 kdaltons); and vitamin B_{12} (1.3 kdaltons).

Figure 1A

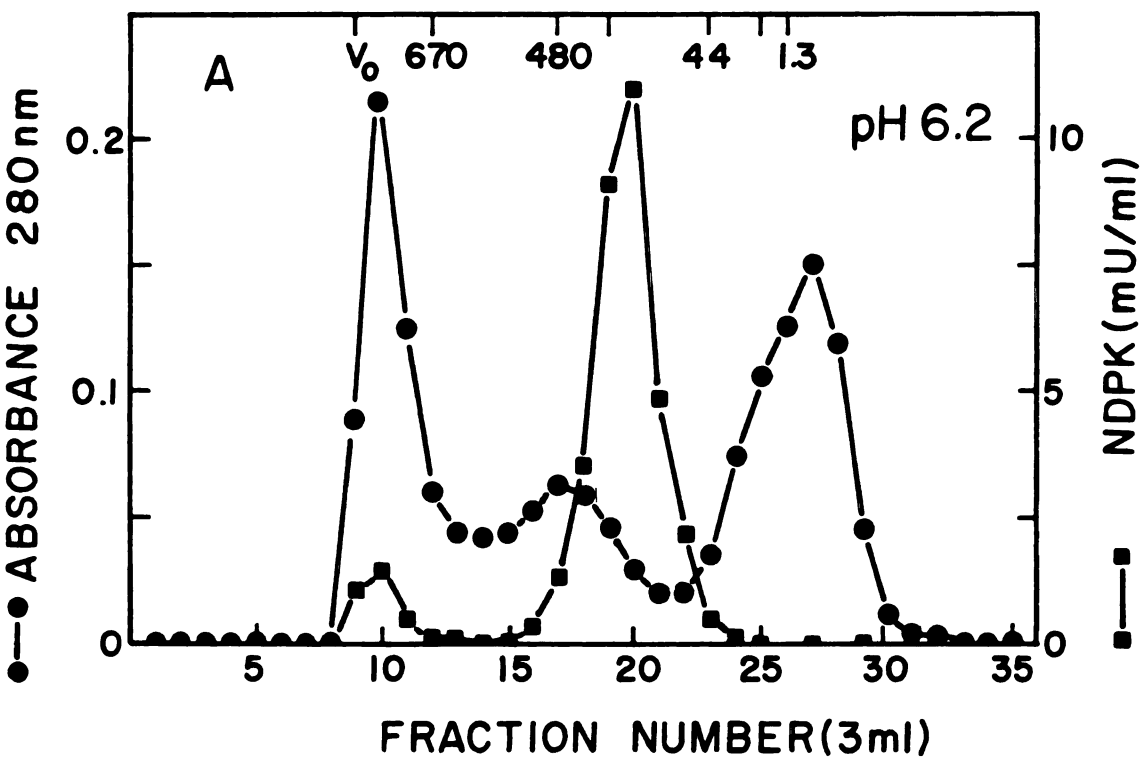

A
V_o
670
480
44
1.3
pH 6.2
ABSORBANCE 280nm
NDPK(mU/ml)
0.2
0.1
0
10
5
0
5
10
15
20
25
30
35
FRACTION NUMBER(3ml)

Figure 1B

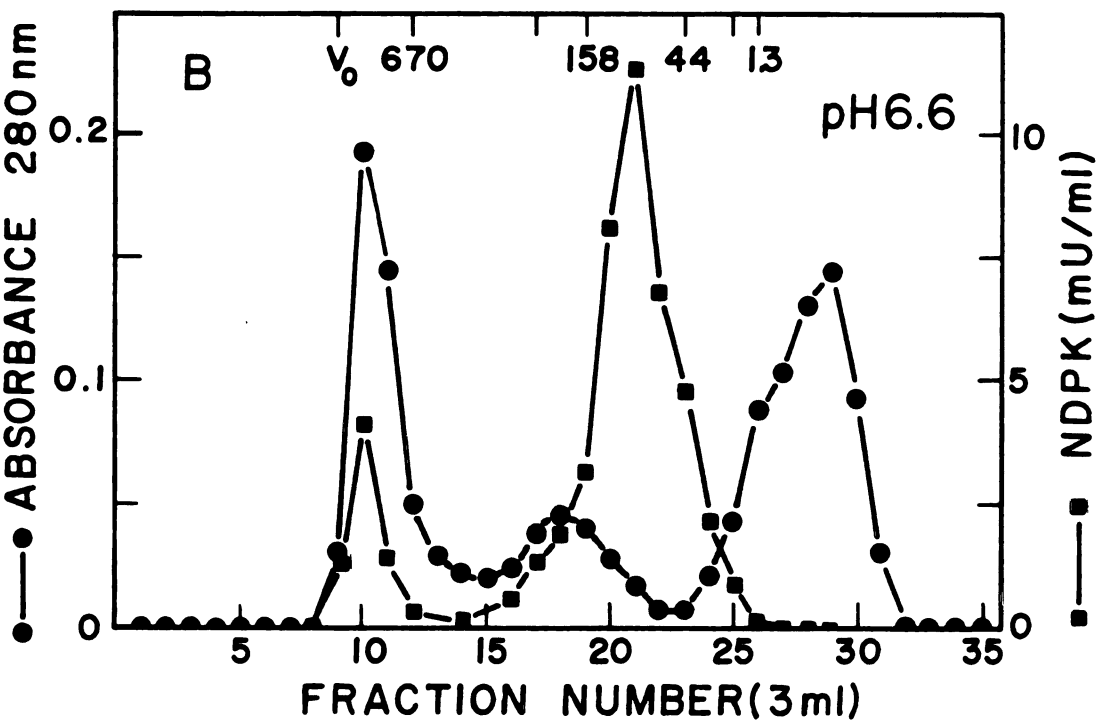
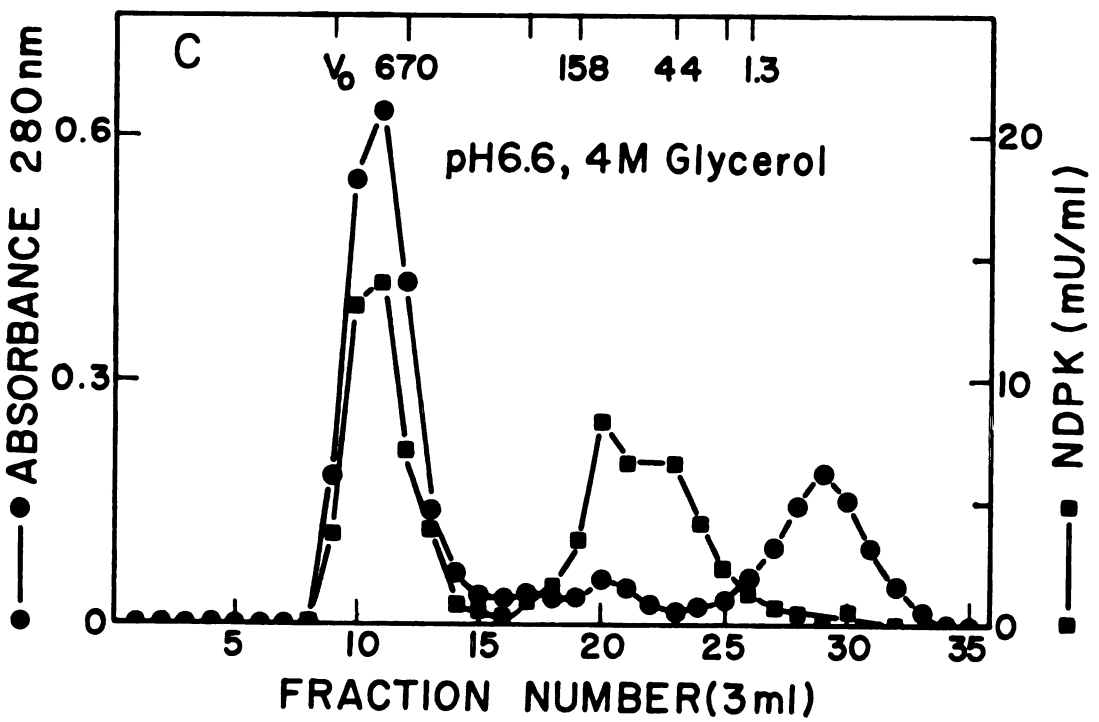

B
V_o 670
158
44
13
pH6.6
ABSORBANCE 280nm
NDPK (mU/ml)
0.2
0.1
0
10
5
0
5
10
15
20
25
30
35
FRACTION NUMBER(3ml)

Figure 1C


C
V_0 670
158
44
1.3
pH6.6, 4M Glycerol
ABSORBANCE 280nm
NDPK (mU/ml)
0.6
0.3
0
20
10
0
5
10
15
20
25
30
35
FRACTION NUMBER(3ml)

(data not shown) and so we conclude that glycerol promotes the association of NDP kinase with rings.

As shown in Figure 1A decreasing the pH to 6.2 decreases the amount of NDP kinase which is ring associated by 50 %. By repeating a gel filtration step twice at pH 6.2, it is possible to almost eliminate NDP kinase activity from oligomeric rings. This may be a convenient technique for removing NDP kinase from assembly-competent microtubule protein.

The Association of NDP Kinase with Microtubules In Vitro

In previous studies (31) we found that in the presence of 6 M glycerol NDP kinase persisted in microtubule protein preparations through 5 cycles of assembly-disassembly, remaining at a constant specific activity through the last 2 cycles. We proposed that NDP kinase might be a microtubule associated protein because of this behavior. More recently Burns and Islam (29) have gel-filtered assembled microtubules on Sepharose 2B columns and shown that microtubules can be separated from NDP kinase activity under these conditions. As a result they have concluded that NDP kinase is not microtubule associated, that it is merely trapped in the microtubule pellets during microtubule protein isolation. To resolve this conflict we have done two types of experiments.

First, we isolated microtubule protein by the method of Sheterline (32) in which microtubules are pelleted

through a cushion of 30 % sucrose at 30°C and 40,000 x g for 60 minutes. This reduces the possibility that NDP kinase is merely being trapped in microtubule pellets. The NDP kinase specific activity of microtubule protein prepared in this way was 0.15 units/mg of protein compared with 0.05 units/mg for 2 cycle microtubule protein prepared as described under "Experimental Procedures", supporting the view that NDP kinase binds to microtubules and remains bound as they pellet through sucrose.

In other experiments we followed the time course of NDP kinase incorporation into microtubules (Figure 2). At various times during <u>in vitro</u> assembly at 30° or 35°C, microtubules were pelleted by 3 minute centrifugations at 175,000 x g in a Beckman Airfuge at room temperature. The amount of protein and NDP kinase activity was determined in the resuspended pellets for each time point. As can be seen in Figure 2, microtubule assembly reached a steady state level by 6 minutes at 35°C and by 8 minutes at 30°C. NDP kinase appeared in the pellets more rapidly; the ratio of NDP kinase activity to the amount of protein in pelleted microtubules decreased after 1 minute. This demonstrated that the incorporation of NDP kinase into microtubules occured early in assembly. This was especially obvious when assembly was slowed down by doing the experiment at 30°C (Figure 2B). The higher NDP kinase activity early

Figure 2. The association of NDP kinase with microtubules during assembly. Samples of microubule protein were incubated with 1 mM GTP in an airfuge rotor that was kept at 30° or 35°C in a water bath. Cold (4°C) microtubule protein was added to GTP immediatly before the sample was warmed in the rotor. At the end of the chosen assembly interval, the rotor was centrifuged at 195,000 x g for 3 minutes at room temperature in the Beckman Airfuge. Supernatants were removed with a syringe and the pellets were resuspended for assay of protein or NDP kinase activity. In the 35°C experiment 110 ul samples assembled contained 5.1 mg/ml microtubule protein and 57 mU NDP kinase/mg), while in the 30°C experiment 150 ul samples contained 3.0 mg/ml microtubule protein with 46 mU NDP kinase/mg.

Figure 2A

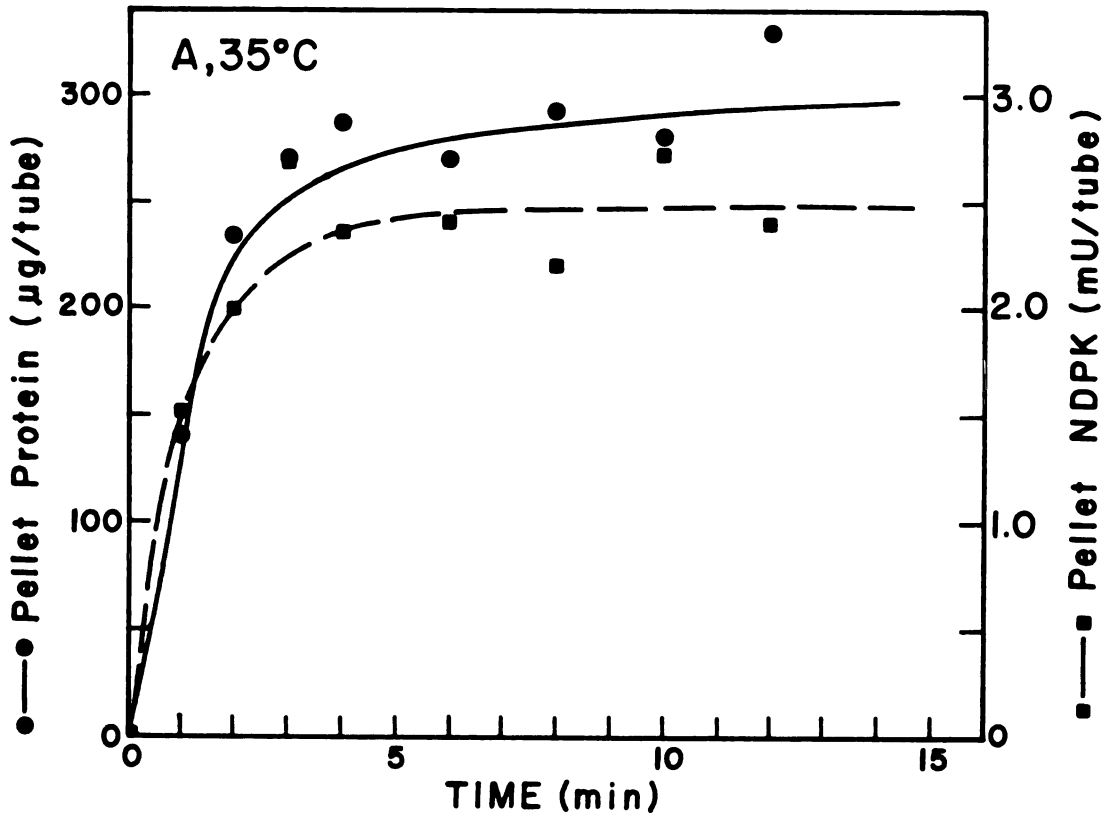
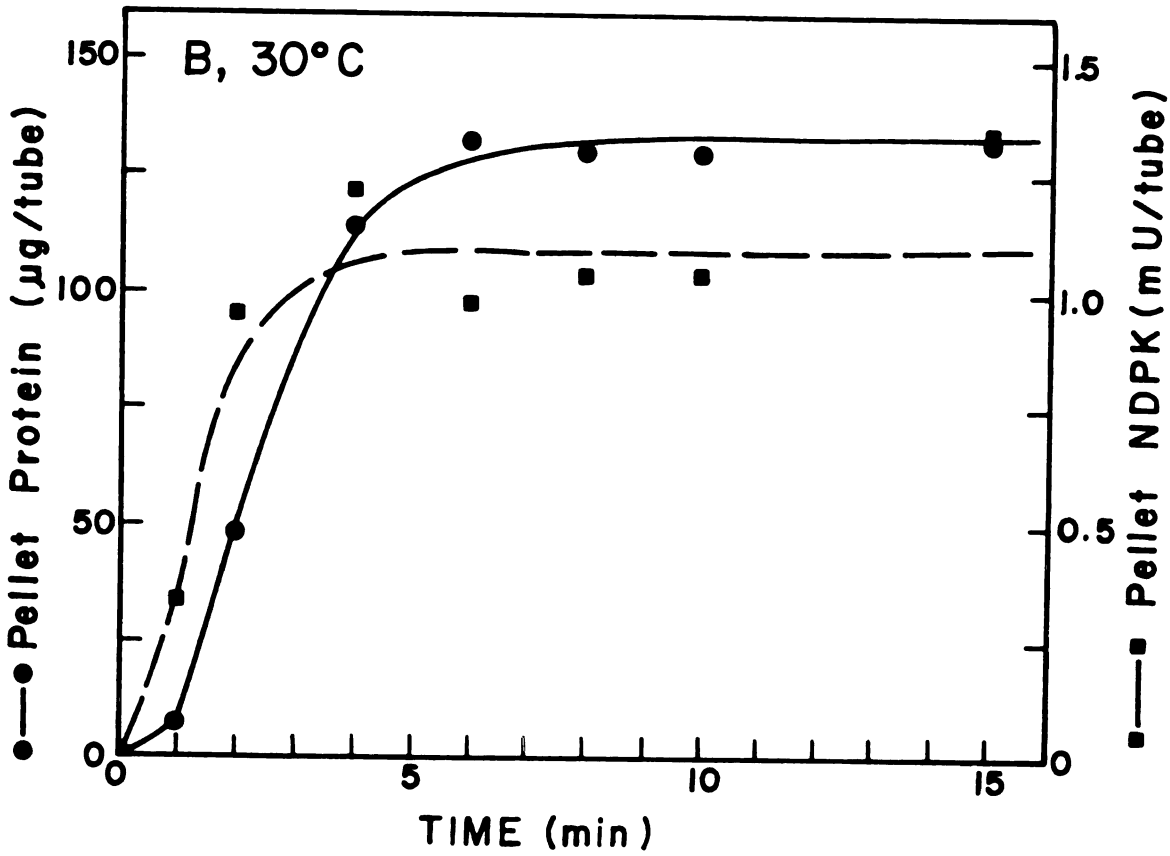

A,35°C
300
200
100
0
3.0
2.0
1.0
0
0
5
10
15
TIME (min)
● Pellet Protein (µg/tube)
■ Pellet NDPK (mU/tube)

Figure 2B


B, 30°C
150
100
50
0
0
5
10
15
1.5
1.0
0.5
0
TIME (min)
●—● Pellet Protein (µg/tube)
■—■ Pellet NDPK (m U/tube)

in assembly suggests that NDP kinase may have a role in initiation and early elongation events during assembly.

The results shown in Figure 2 suggest that NDP kinase is binding to microtubules, but there are still two alternative explanations of the data which must be eliminated in order to validate that conclusion. The first possibility is that NDP kinase is aggregating and pelleting independently of microtubule formation. The second possibility is that NDP kinase is merely being trapped in the microtubule pellets without actually binding to microtubules. The experiment summarized in Table I was performed in order to examine these two possibilities.

Microtubules were assembled at 35^{o}C with 2 mM GTP for 0 to 10 minutes after which they were pelleted, as before, by 3 minute centrifugations at 175,000 x g in the Beckman Airfuge at room temperature. After 10 minutes of assembly, 228 ug of protein and 1.8 mU of NDP kinase activity were pelleted. When 83 uM colchicine was included in the assembly buffer, no protein and no NDP kinase activity could be pelleted. If NDP kinase was aggregating and pelleting independently of microtubules, then we would not expect colchicine, which prevents microtubule assembly, to affect the amount of pelleted NDP kinase. From this experiment we conclude that the increase in pellet NDP kinase activity which parallels microtubule assembly is dependent upon microtubule

TABLE I

Cosedimentation of NDP kinase with Microtubules

This experiment was done as described in Figure 2 at 35°C. The samples had a microtubule protein concentration of 5.0 mg/ml and 0.242 U/ml of NDP kinase. Some samples contained 0.25 uCi/ml [^{14}C]BSA (bovine serum albumin). The values shown are the means +/- standard deviations for triplicate determinations.

Incubation Time (min)	Addition	Net Protein or Activity in Pellets[a] ug Protein	mU NDP kinase	cpm BSA	NDP kinase Recovery
0		0 ± 1.2	0 ± 0.06	-	93%
10		228.3 ± 6.0	1.83 ± 0.05	-	86%
0	83 uM colchicine	-13.3 ± 0.6[b]	0.03 ± 0.03[b]	-	
10	83 uM colchicine	-0.3 ± 4.4[b]	-0.19 ± 0.18[b]	-	80%
with [^{14}C]BSA					
0		1.3 ± 1.7	0.16 ± 0.06	0 ± 9.1	105%
10		225.2 ± 4.1	2.23 ± 0.31	215. ± 24.5	100%
10	83 uM colchicine	4.3 ± 1.3	0.23 ± 0.18	3.0 ± 35.0	

Incubation Time (min)	Addition	% of Total Protein or Activity in Pellets[c] Protein	NDP kinase	BSA radioactivity
0		0.3 ± 0.3	0.7 ± 0.3	0
10		45.1 ± 0.8	9.2 ± 1.2	2.5 ± 0.5
10	83 uM colchicine	0.9 ± 0.3	1.0 ± 0.7	0

a. All values represent the net protein or activity above that for the samples incubated for 0 minutes without colchicine/
b. Negative values are caused by values that are smaller than those for the blanks.
c. These values are the net amount of protein or activity in the pellets as a percentage of the total amount in the sample.

formation.

In the second part of the experiment (Table 1B) we included 1.25 ug / ml [^{14}C] bovine serum albumin in the assembly mixture. Since bovine serum albumin does not bind to microtubules, the amount of [^{14}C] serum albumin that could be pelleted gave us a quantitative estimate of the amount of soluble protein trapped in the microtubule pellets. If there was any association of serum albumin with microtubules this value would be an overestimate, so that the percent of serum albumin found in the pellets is a maximum estimate or upper limit to the percent of soluble protein that might be trapped in the pellets without binding. As shown in Table 1B, after 10 minutes of assembly, 2.5 % of the serum albumin was recovered in the microtubule pellets. If NDP kinase did not bind to microtubules we would expect that 2.5 % or less of the NDP kinase in the samples would be recovered in the pellets. In fact 9.2 % of the sample NDP kinase was found in the pellets. We believe, therefore, that NDP kinase is binding to and pelleting with microtubules.

<u>The Association of NDP Kinase with Microtubules in 3T3 Cells</u> - In experiments such as those shown above we and others have sought to determine whether NDP kinase binds to microtubules and to other microtubule protein structures <u>in vitro</u>, but what we really want to know is whether NDP kinase binds to microtubules in the cell. We

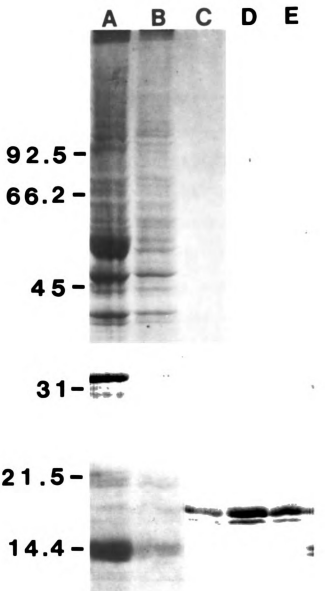
decided to attempt indirect immunoflourescent staining of cells for NDP kinase. This required that we produce an antibody against NDP kinase. Our attempts to produce an antiserum against bovine brain cytosol NDP kinase isolated by the method of Nickerson and Wells (16) were unsucessfull so we isolated the particulate fraction NDP kinase from bovine brain as discussed under "Experimental Procedures" and were able to produce rabbit anti-serum to this enzyme which is distinct from but similiar to the microtubule NDP kinase (16).

As shown in Figure 3, SDS-PAGE examination of the isolated NDP kinase antigen showed it to be a homogeneous doublet of bands with molecular weights of about 18,000. Robinson et al. (20) using a very similiar isolation procedure detected only one type of subunit in particulate NDP kinase, but they were using a SDS-PAGE system with inadequate resolving power to see the doublet. Our gels were 9-16 % linear gradient gels with an acrylamide to bis-acrylamide ratio of 19 used with the Laemmli (23) system of buffers. We have previously reported (16) that the NDP kinases from bovine brain microtubule protein and from bovine brain cytosol examined with this same SDS-PAGE system also contained two different subunits with molecular weights of about 18,000.

Rabbit anti-NDP kinase serum was used for the isolation of rabbit anti-NDP kinase IgG by chromatography

Figure 3. SDS-PAGE of bovine brain particulate NDP kinase. Samples from each step in the isolation procedures were electrophoresed on 9-16 % gradient gels as described under "Experimental Procedures". Lane A is the bovine brain first supernatant. Lane B is the Brij 56 extract from the pellets of the first centrifugation step. Lane C is the NDP kinase eluted from Blue Sepharose. Lane D is the Blue Sepharose NDP kinase containing fractions concentrated with a CX-30 ultrafilter. Lane E is NDP kinase after chromatography on TSK 55s. This is the NDP kinase which was used as an antigen for antibody production. NDP kinase consists of a doublet of bands with molecular weights of about 18,000 daltons.

Figure 3



on Protein A Sepharose. As shown in Figure 4A, anti-NDP kinase activity was present in both serum and isolated IgG, but it was absent in preimmune serum.

The antibody had been raised against a form of bovine brain NDP kinase which is unlikely to associate with microtubules since it is membrane associated (20). As shown in Figure 4B, the antibody which had been raised to bovine brain particulate NDP kinase cross-reacted with the bovine brain microtubule NDP kinase isolated from cytosol by the method of Nickerson and Wells (16). Thus, we would expect the antibody to recognize the form of NDP kinase that may associate with microtubules.

We wanted to use an antibody raised against bovine brain NDP kinase to stain mouse fibroblasts, so it was important to see whether our IgG would cross-react with NDP kinase from these cells. A flask of 3T3 cells was homogenized and 150 ug of the homogenate was loaded on a gel for SDS-PAGE. After electrophoresis, the separated proteins were blotted onto nitrocellulose and the blot was probed with anti-NDP kinase IgG as described in "Experimental Procedures". As shown in Figure 4C, only a band of about 18,000 daltons is recognized by our antibody, suggesting that, in 3T3 cells, our antibody is monospecific for NDP kinase. It is important to note that with this SDS-PAGE system and blotting protocol the ability to resolve the NDP kinase doublet would be lost, so we do not know whether 3T3 cell NDP kinase has one or

Figure 4. Characterization of anti-NDP kinase IgG.

A. On this Ouchterlony plate the center well (C) contained 20 ul of NDP kinase (1.5 mg/ml). Well 1 contained 20 ul of preimmune serum. Well 2 contained 30 ul anti-NDP kinase serum. Well 3 contained 20 ul of anti-NDP kinase IgG (0.6 mg/ml). Well 4 contained an affinity labeled anti-NDP kinase which was not used in this study because it was only produced with very small yields. This plate shows that the anti-NDP kinase antibody was present in both immunized rabbit serum and in the IgG isolated from that serum.

Figure 4A

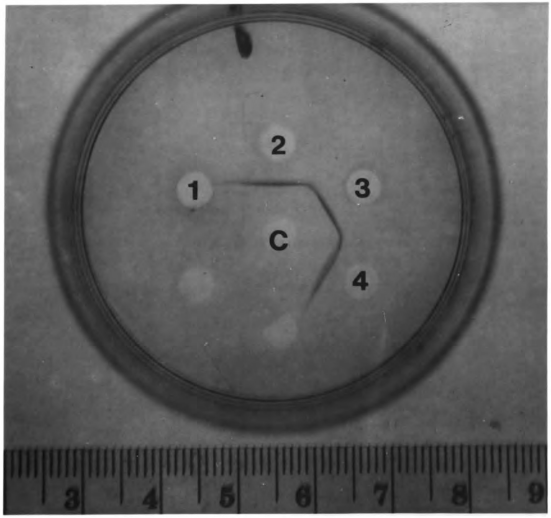


Figure 4. Characterization of anti-NDP kinase IgG.

B. This immunoblot was stained with anti-NDP kinase IgG. Samples were prepared for electrophoresis by treatment with 20 mM iodoacetamide and then denaturation in sodium dodecyl sulfate. Samples to be electrophoresed on Gel A were supplemented to 100 mM with -mercaptoethanol before electrophoresis while the samples for gel B were not reduced. After electrophoresis, the protein bands were blotted onto nitrocellulose and stained with anti-NDP kinase IgG as described under "Experimental Procedures".

Lane 1. Microtubule NDP kinase isolated from bovine brain cytosol as described by Nickerson and Wells (16).

Lane 2. NDP kinase isolated form the particulate fraction of bovine brain as described under "Experimental Procedures". This is the antigen that was used to produce the anti-NDP kinase IgG.

Figure 4B

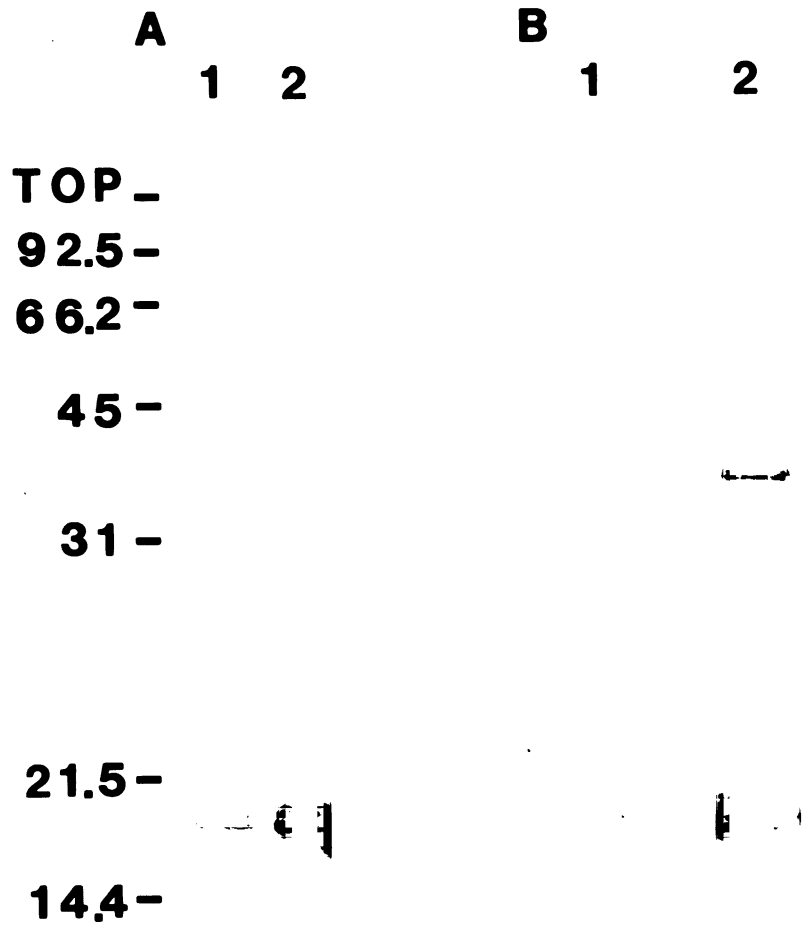
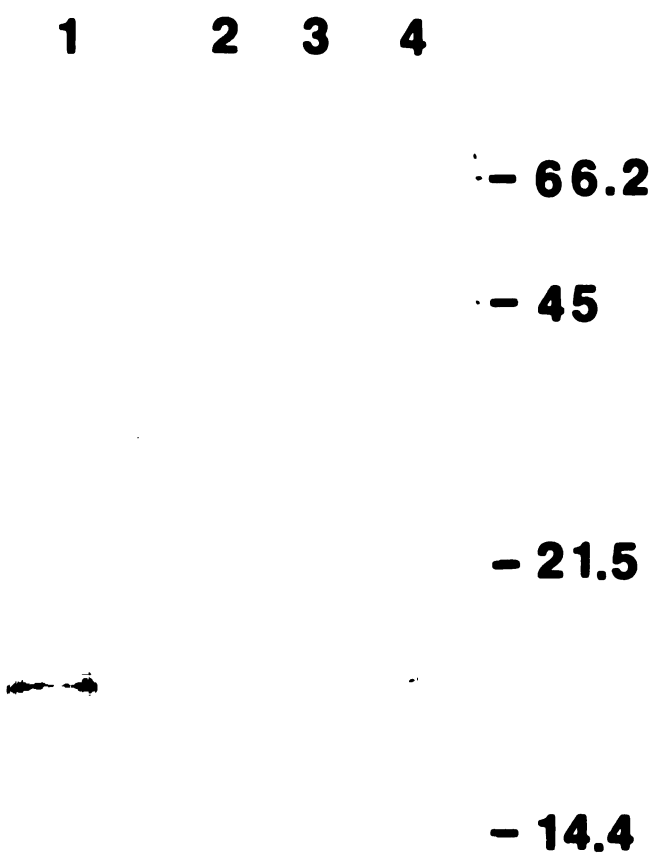


Figure 4. Characterization of anti-NDP kinase IgG.

C. This immunoblot was prepared and stained with anti-NDP kinase. Lane 1 contained 2 ug of the antigen, bovine brain NDP kinase; lane 2 contained 2 ug of actin; lane 3 contained 2 ug of microtubule protein; lane 4 contained 100 ug of 3T3 cell homogenate. From this experiment we can conclude that our anti-NDP kinase IgG was, in 3T3 cells, monospecific for NDP kinase and we can conclude that our antibody did not recognize either actin or the cytoskeletal proteins in 2 cycle microtubule protein. The standards for this gel were lysozyme (14.4 kdaltons), soybean trypsin inhibitor (21.5), ovalbumin (45), and bovine serum albumin (66.2).

Figure 4C



two distinguishable subunits. The same experiment was done with isolated microtubule protein and with purified actin. As can be seen in Figure 4C, neither actin nor any microtubule protein present in sufficient quantity can be recognized by our anti- NDP kinase IgG. If 3T3 cell microtubules are stained by this antibody, we know that it is NDP kinase that is being stained and not other microtubule proteins.

The use of anti-NDP kinase for the staining of 3T3 cells is shown in Figure 5. Anti-NDP kinase serum and IgG stain filamentous structures in the cell which appear to radiate from the nucleus. The staining is very dependent on antibody concentration. At low concentrations, there is little staining, while at high concentrations the background stains too intensely to see filaments. An optimal concentration of antibody must be determined for each batch of serum or IgG. The staining pattern for NDP kinase is distinctly different from the pattern of actin localization, so we can be sure that NDP kinase is not associated with actin filaments. NDP kinase localization is similiar to tubulin localization in 3T3 cells except that there is less cytoplasmic staining so that the filaments stand out more clearly against the background. This may be caused by the presence of a larger percentage of unpolymerized tubulin in cytoplasm then the percentage of non-filamentous NDP kinase.

Figure 5. Indirect immunofluorescent staining of 3T3 cells for NDP kinase and cytoskeletal proteins.

Indirect immunofluorescent staining was done as described in "Experimental Procedures". The bar on each picture represents 1 um.

A. In this picture the 3T3 cell was stained with preimmune serum.

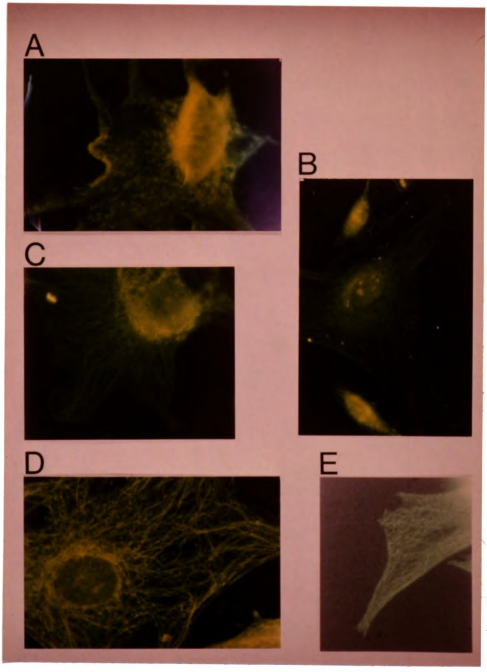
B. This 3T3 cell was stained with a 1:100 dilution of rabbit anti-actin serum.

C. This shows the staining of a 3T3 cell with a 1:100 dilution of rabbit anti-tubulin serum.

D. This 3T3 cell was stained with a 1:50 dilution of rabbit anti-NDP kinase serum.

E. This shows the staining of a 3T3 cell with 50 ug/ml anti-NDP kinase IgG.

Figure 5



When cytoskeletons were extracted by the method of Osborn et al. (33) before protein fixing, microtubules remained intact but all NDP kinase staining was lost. We believe that the Triton X-100 in the extraction buffer breaks down the interaction of NDP kinase with microtubules. Neither microtubules nor filamentous NDP kinase could be detected in cells which had been incubated in 100 uM colchicine in tissue culture medium for 10-30 minutes before the cells were fixed (data not shown).

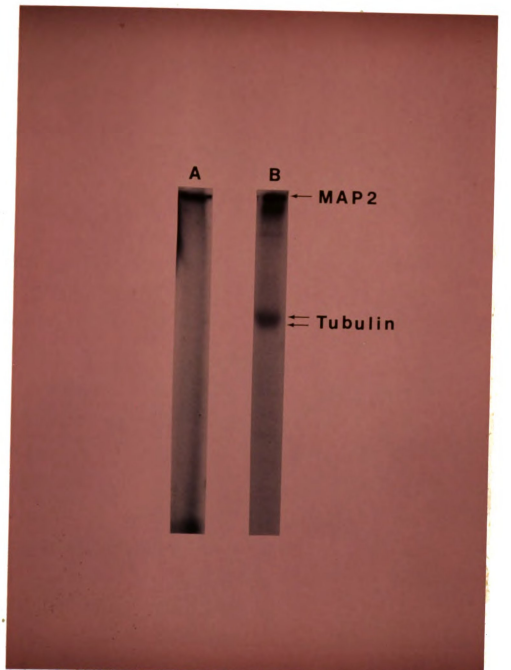
These immunochemical localization studies suggest that NDP kinase may be associated with microtubules in 3T3 cells but, as discussed below, this must be treated as a preliminary result. We would like to improve our localization techniques my microinjection studies with fluoescently labeled NDP kinase.

Transphosphorylation Catalyzed by NDP Kinase - As had been reported by Maccioni and Seeds (26) tubulin could be affinity labeled with oxGTP. This tubulin was capable of undergoing a cycle of assembly-disassembly after which the covalently attached E-site nucleotide should be oxGDP. We were able to achieve a stoichiometry of about 0.4 moles of oxGDP incorporated per mole of tubulin. This is substantially below the theoretical maximum of 1 mole per mole of tubulin, but it was good enough for use

Figure 6. Transphosphorylation of oxGDP labeled tubulin

Samples of microtubule protein or oxGDP affinity labeled microtubule protein prepared as described under "Experimental Procedures" were brought to 20 mM PIPES, pH6.6, 0.8 mM $MgCl_2$ and 75 ug/ml of bovine brain NDP kinase was added. [gamma-^{32}P] ATP was added to a final concentration of 1 uM and the sample was incubated for 3 minutes at 30^{o}C. The incubation was terminated by the addition of SDS-PAGE sample buffer and the sample was electrophoresed with BioRad low molecular weight standards. An autoradiographs was prepared from the dried gel from which a lane of standards had been cut and stained. Lane A shows the phosphorylation of microtubule protein. The only phosphorylated band detected was the high molecular weight protein MAP2 which is a substrate for the endogenous protein kinase. Lane B shows the phosphorylation of microtubule protein which had been affinity labeled with oxGDP. Phosphate is incorporated into the tubulin bands for this sample even though, as seen in Lane A, tubulin is not a substrate for the protein kinase. We suggest that the phosphate is being incorporated into the covalently bound nucleotide on tubulin.

Figure 6



in transphosphorylation experiments performed as discussed below.

We incubated microtubule protein or affinity labeled microtubule protein with [gamma-^{32}P] ATP and bovine brain NDP kinase for 3 minutes, after which the sample was treated with SDS-PAGE sample preparation buffer and then electrophoresed in our SDS-PAGE system as discussed in "Experimental Procedures". After electrophoresis the gels were dried and autoradiographs were prepared. Any nucleotide which was not covalently attached to the tubulin molecule is released by treatment with SDS and migrates slightly ahead of the dye front in our SDS-PAGE system. When [^{3}H] oxGTP was used for the affinity labeling of tubulin and the tubulin was not treated with [gamma-^{32}P] ATP, the tubulin bands could be cut out of the gel and counted for ^{3}H to estimate the amount of affinity label incorporated.

The microtubule protein preparations used in this experiment contain a protein kinase activity which is known (34-36) to phosphorylate the high molecular weight microtubule associated protein MAP2. As shown in Figure 6, MAP2 is the only protein which incorporates labeled phosphate in normal microtubule protein. When the tubulin molecules in the same preparations are affinity labeled with oxGDP, tubulin also incorporates labeled phosphate. Since tubulin is, as we can see, not a substrate for the endogenous protein kinase, we can

conclude that the ^{32}P label is incorporated into the covalently attached nucleotide. Since that nucleotide is covalently attached, we know that it did not exchange onto tubulin, but that it must have been phosphorylated by ATP without ever leaving it's tubulin binding site. This phosphorylation of tubulin bound nucleotide suggests that a true transphosphorylation mechanism exists, catalyzed by the NDP kinase added to this preparation. We are currently using this experimental design to explore the nature of the transphosphorylation mechanism.

DISCUSSION

There are two mechanisms by which E-site GTP might be generated to support microtubule assembly: nucleotide exchange and the transphosphorylation of E-site GDP to GTP without exchange.

Exchange:

$$\text{Tubulin-GDP} + {}^{*}\text{GTP} \quad \text{Tubulin-}{}^{*}\text{GTP} + \text{GDP}$$

Transphosphorylation:

$$\text{Tubulin-GDP} + {}^{*}\text{NTP} \quad \text{Tubulin-GTP} + {}^{*}\text{NDP}$$

A transphosphorylation mechanism could be driven by any nucleoside triphosphate (NTP) as pictured above.

Under conditions of pH, ionic strength, and metal ion concentration favoring assembly, ATP and every other nucleoside triphosphate tried will induce microtubule assembly (1,30,37,38), even though binding of these nucleotides to tubulin cannot be detected. Jacobs and his colleagues (38,39) were the first to suggest that a transphorylase activity in microtubule protein preparations transfers the gamma phosphate of NTP to a tightly bound tubulin-GDP, leaving a GTP bound to the tubulin E-site. This tubulin-GTP would then be capable

of participating in assembly reactions requiring GTP.

It has been suggested that the proposed transphosphorylation reaction might be catalyzed by a nucleoside diphosphate kinase (1,2).

Penningroth and Kirshner (1) demonstrated that microtubule protein preparations contain a true transphorylase activity phosphorylating tubulin E-site bound GDP in situ. They incubated tubulin containing bound GDP with a mixture of [^{3}H] GTP and [gamma-^{32}P] UTP under conditions that would support assembly. They found that the ^{32}P labeled nucleotide appeared on the tubulin dimer almost 10 times faster than the ^{3}H labeled nucleotide and concluded that transphosphorylation is occuring 10 times faster than simple nucleotide exchange. The validity of this experiment has been challenged by Brylawski and Caplow (15) who have suggested that it is impossible to remove free nucleotide from microtubule protein preparations and so the results of the double label experiment are complicated by isotope dilution. Jacobs and Huitorel (2) have performed a similiar double label experiment with [^{3}H] GDP and [gamma-^{32}P] GTP. Tubulin was preloaded with [^{3}H] GDP and then incubated with [gamma-^{32}P] GTP. The nucleotide bound to the E-sites on dimers was almost all ^{32}P labeled while the E-sites on 36s rings was both ^{3}H and ^{32}P labeled. This experiment suggests that E-site GTP on dimers is produced by

exchange while E-site GTP on rings is produced by transphosphorylation. This result cannot be dismissed as an artifact of isotope dilution, but, does not finally settle the debate. NDP kinase is associated with the 36s rings and for reasons of simple propinquity may preferentially phosphorylate GDP being released from nearby ring E-sites.

In this paper we report an experimental protocol that will permit the unambiguous study of transphosphorylation. We have affinity labeled the E-site of tubulin in assembly competent microtubule protein with an analogue of GDP. If this GDP can be phosphorylated to GTP it must be done by a transphosphorylation mechanism since the nucleotide is covalently attached.

We have taken advantage of the affinity labeling procedure of Maccioni and Seeds (26), who have shown that oxGTP affinity labels the E-site of tubulin specifically and that the resulting complex is assembly competent. By taking this tubulin through an assembly-disassembly cycle we are able to generate a tubulin dimer affinity labeled with oxGDP. As shown in Figure 6, microtubule protein preparations contain a protein kinase that phosphorylates principly MAP2. Tubulin could not be phosphorylated by [gamma-^{32}P] ATP but oxGDP affinity labeled tubulin could. From this experiment we conclude that the tubulin protein is not being phosphorylated, since the endogenous

protein kinase is not using tubulin as a substrate, so the phosphorylation of the tubulin is due to phosphorylation of the affinity bound nucleotide. This shows that a transphosphorylation mechanism exists since the covalently bound nucleotide cannot be exchanging with nucleotide in solution. More intensive studies using this protocol should allow us to study the kinetics and mechanism of this mechanism.

In our experiments (Figure 1), we found that a maximum of 15 % of the NDP kinase in microtubule protein preparations was associated with rings, but that glycerol could increase this to 50 %. The association of NDP kinase with rings is an important phenomena since Jacobs and Huitorel (2) have shown that transphosphorylation, if it occurs, occurs on the rings.

Preparations of microtubule protein which are made by cycles of assembly-dissassembly contain tubulin in two forms, as 6s dimers and as ring-shaped oligomers of 30-36s (this is reviewed in 40). Oligomeric microtubule protein rings also contain MAPs, especially the high molecular weight MAP1 and MAP2, and differ in structure according to whether the microtubule protein was prepared in the presence or in the absence of glycerol (40,41). Microtubule protein prepared in the presence of glycerol contains rings that are 36s double-walled structures (40,41), while microtubule protein prepared in the absence of glycerol contains 18s rings and 30s rings

which resemble two stacked 18s rings (40,43). These 18s and 30s rings are interconvertable and exist in equilibrium. Typically, 25-75% of the protein in a microtubule protein preparation isolated by two cycles of assembly exists in oligomeric rings, depending on solution conditions and protein concentration. It may be that NDP kinase associates with the 36s rings which are present in the presence of glycerol, but not with the 30s rings present without glycerol. There are two views that we might take of the glycerol dependence of NDP kinase-ring association. First, the high degree of ring association may be artifactually caused by glycerol concentrations that do not exist in vivo. Second, glycerol may mimic the very high protein concentrations which exist in vivo, but which are not achievable in in vitro experiments. We are inclined to accept the second interpretation of these results, since if a transphosphorylase activity actually exists and if it acts on ring tubulin, the association of NDP kinase with rings might be expected.

The tubulin of rings and the tubulin that exists as free dimers are not quickly interconverted, nor are they in a simple dimer-ring equilibrium (reviewed in 40); the 6s and 36s forms of tubulin did not interconvert even during a complete cycle of assembly-disassembly (44). Other studies by Zeeberg et al. (45) and by Pantaloni et al. (46) show that there is a very slow exchange of

subunits between 36s rings and 6s dimers. 36s rings are direct cold induced disassembly products of microtubules; they are not formed from microtubule released dimers (45), but once rings and dimers are produced by cold disassembly they exchange subunits with a half time of about 90 minutes (46).

Studies of in vitro microtubule assembly by synchrotron radiation scattering have shown that oligomeric rings are not nucleation centers for assembly; rather, they are disassembled into protofilament fragments which nucleate assembly (47-49). The tubulin subunits which appear in microtubules during the early stages of assembly come preferentially from 36s rings (46). A transphosphorylation mechanism for the generation of E-site GTP would be more important for tubulin in rings because, as we have seen, the nucleotide on ring tubulin is not readily exchangeable and dimer and ring forms of tubulin do not readily exchange.

In previous studies (16) conducted in the presence of glycerol, we found that NDP kinase associated with microtubules in vitro. NDP kinase co-persisted with microtubules through 5 cycles of assembly-disassembly and co-sedimented with assembled microtubules through sucrose gradients. We wanted to confirm this association in the absence of glycerol and by a protocol that would be more useful for further studies. To this end microtubules, were assembled in 150 ul Airfuge tubes and pelleted at

various times during assembly by very rapid (1-3 minute) centrifugations. This study was only made possible by the very high g forces and small dimensions of the Beckman Airfuge, which allowed us to freeze the action very quickly at various times during assembly. As seen in Figure 2, the NDP kinase content of microtubules was highest early in assembly and decreased during the later elongation stage. This implies that if NDP kinase plays a role in assembly, it does so during initiation or early elongation events.

To rule out the possibility that NDP kinase was aggregating and pelleting in a microtubule independent manner we showed that colchicine prevented the pelleting of both microtubule protein and NDP kinase (Table I). Since NDP kinase might be trapped in the microtubule pellets without binding to microtubules, we did an experiment to obtain an upper limit estimate of the amount of such trapping that might occur (Table I). Microtubules were assembled and pelleted in the presence of [^{14}C] bovine serum albumin. Only 2.5 % of the serum albumin was recovered in the microtubule pellets, while 9.2 % of the NDP kinase was pelleted. From this experiment we would expect that 2.5 % or less of the total NDP kinase would be trapped, so we conclude that the rest of the pellet NDP kinase was microtubule bound.

The stoichiometry of NDP kinase binding to microtubules was very low. It may be that we have not found optimal conditions for binding.

We have proposed that NDP kinase catalyzes a transphosphorylation reaction generating tubulin E-site GTP. There is nothing about this proposal that requires NDP kinase to bind to microtubules once they have been assembled. Nevertheless, NDP kinase does bind to microtubules in vitro. It seemed important, therefore, to determine whether NDP kinase might bind to microtubules in vivo. This was done by indirect immunofluorescent staining of 3T3 cells with rabbit anti-NDP kinase IgG.

As seen in Figure 5, antibody against NDP kinase decorates filaments in the cytoplasm of 3T3 cells. These filaments originate near the nucleus. They are not observed in cells that are pretreated with colchicine, which suggests that they are microtubules or that they depend on microtubules for structure. The antibody against NDP kinase used in this study was specific for NDP kinase (Figure 4). It did not recognize tubulin or any other 3T3 cell protein. We conclude that NDP kinase is associated with filamentous structures in 3T3 cells that are most likely microtubules.

Immunofluorescent staining studies are not an ideal way to localize proteins. It may be that fixation procedures in organic solvents artifactually condenses

proteins into filamentous structures and it is probable that fixation changes the availability of various antigenic determinants for antibody binding. Despite these difficulties, immunochemical localization may be the best available technique for locating proteins in the cell. We are contemplating microinjection studies with fluorescently tagged NDP kinase to confirm that this enzyme is microtubule associated in the cell. We believe that the use of both techniques together solves the problems of validity that each technique presents alone.

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